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Means and methods for treating arthitis

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Means And Methods For Treating Arthritis

The present invention relates to the use of at least one inhibitor of at least one ABC-transporter capable of transporting hyaluronan across a lipid bilayer, for the preparation of a pharmaceutical composition for the treatment of arthritis. Furthermore, the present invention relates to a method for screening a compound which is suitable for the treatment of arthritis. The present invention also relates to a method for screening a compound which reduces the transport of hyaluronan mediated by (an) ABC-transporter(s). Furthermore, the present invention relates to a method for identifying a subject at risk for arthritis as well as to a method of screening for a compound which is suitable for the treatment of arthritis in a subject. In addition, the present invention relates to a method of preventing, ameliorating and/or treating the symptoms of arthritis in a subject.

A variety of documents is cited throughout this specification. The disclosure content of said documents (including any manufacturer's specifications, instructions etc.) is herewith incorporated by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

Arthritis is a very common disease: its prevalence is increasing with age and is 9% at an age of 20 years, 17% at 35 and 90% over 65. Symptoms are common at an age between 50 and 60. The pathogenesis is favoured by several risk factors: genetical disposition, joint injury, obesity, joint deformity, local biomechanical factors and inflammation. The total annual costs for medial treatment and economical loss caused by disability has been calculated for Germany to amount to 12 billion €. No other disease has a larger pharmaceutical market.

Arthritis is accompanied with a loss of cartilage at the joint surface. The cartilage goes through different stages during pathogenesis. At first chondrocytes try to

replace loss of cartilage by increased synthesis and proliferation; simultaneously lacunae of edema and increased water binding occurs. Increased water binding leads to softening of the cartilage matrix. The cause of edema and water accumulation is known form other systems: an increased hyaluronan production. These phenomena are observed before fibrillation or cartilage erosion. At the second stage new cartilage production cannot compensate for the loss and at the third stage loss of cartilage is complete.

The cartilage matrix consists of two main components: type II collagen and high molecular weight aggrecan. Aggrecan is a proteoglycan and is composed of a core-protein that binds many molecules of chondroitin sulfate and keratan sulfate. These proteoglycans decorate a backbone of hyaluronan like a bristles of a bottle brush. Hyaluronan itself is anchored in the membrane of chondrocytes at the membrane integrated synthase. It is further bound by the cell surface receptor CD44.

It is known that the CD44 receptor transmits signals into the cell upon binding to oligosaccharides of hyaluronan and causes increased production of metalloproteases [19]. Therefore it appears likely that an enhanced hyaluronan concentration in osteoarthritic cartilage has a similar effect and also induces protease production.

In spite of this knowledge, academic and industrial research spends enormous efforts for the development of protease inhibitors for treatment of arthritis. Since the discovery of collagenase 1962 almost every large pharmaceutical company has had a protease inhibitor program. Although there were some partial benefits in animal models [20], there is still not a single approved drug and the therapeutic success of protease inhibitors were very sobering [21]. This is not surprising, because protease inhibitors do not inhibit the primary process.

The consequence of joint injury has some similarity with the swelling of a lump after contusion. Also here hyaluronan production is the primary process accompanied by water accumulation that precedes the activation of proteases and other

inflammatory reactions. The swollen tissue enables the invasion of leukocytes and inflammation.

Up to now no therapy exists that can alter the course of the disease or can repair existing damages. Treatment is confined to pain relieve by physiotherapy, analgetic or anti-inflammatory drugs or intraarticularly applied hyaluronan. Intra-articular administration of hyaluronan has been used in animals and man. In man, hyaluronan is being used to relieve pain and improve joint mobility in the treatment of arthritis with intra-articular injections of Hyalgan ® (Sanofi Pharmaceuticals), Orthovisc ® (Anika Therapeutics), and SynVisc ® (Biomatrix, now Genzyme). It has also been proposed for several degenerative joint diseases as an alternative to the traditional steroid therapy [32-34]. However, the benefit of hyaluronan injections remains controversial [1].

Thus, the technical problem underlying the present invention was to provide means and methods for treating and/or preventing arthritis.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Hyaluronan is present in all vertebrates and also in the capsule of some pathogenic bacteria such as Streptococci and Pasteurella. It is a component of extracellular matrices in most tissues and in some tissues it is a major constituent. The concentration of hyaluronan is particularly high in rooster comb (7.5 mg/ml), in the synovial fluid (3-4 mg/ml), in umbilical cord (3 mg/ml), in the vitreous of the eye (0.2 mg/ml) and in skin (0.5 mg/ml). In other tissues that contain less hyaluronan, it forms an essential structural component of the matrix. In cartilage it forms the aggregation centre for aggrecan, the large chondroitin sulfate proteoglycan, and retains this macromolecular assembly in the matrix by specific hyaluronan-protein interactions. It also forms a scaffold for binding of other matrix components around smooth muscle cells on the aorta and on fibroblasts in the dermis of skin. The largest deposit of hyaluronan resides in the skin with about 8 g of an adult human. Hyaluronan has also been detected intracellularly in proliferating cells.

Hyaluronan consists of basic disaccharide units of D-glucuronic acid and D-N-acetylglucosamine. They are linked together through alternating beta-1,4 and beta-1,3 glycosidic bonds. The number of repeat disaccharides, in a completed hyaluronan molecule can reach 10,000 or more and a molecular mass of ~4 million daltons (each disaccharide is ~400 daltons). In a physiological solution, the backbone of a hyaluronan molecule is stiffened by a combination of the chemical structure of the disaccharide, internal hydrogen bonds, and interactions with solvent. A hyaluronan molecule assumes an expanded random coil structure in physiological solutions which occupies a very large domain. The actual mass of hyaluronan within this domain is very low, and ~0.1% molecules would overlap each other at concentrations of 1 mg hyaluronan per ml or higher.

Many different functions have been assigned to hyaluronan. They can be grouped into cellular, physiological and pathological functions. Most of the functions are determined by the physical properties or by interactions with hyaluronan binding proteins. A prominent physiological function of hyaluronan is the creation of hydrated pathways that allow the cells to penetrate cellular and fibrous barriers. Such hydrated pericellular matrices are not only required for cell rounding in mitosis, but also for cell migration during morphogenesis and wound healing. In cartilage hyaluronan is the key element that holds the proteoglycans together to form large aggregates. A hyaluronan binding domain at the C-terminus of aggrecan is responsible for this aggregation.

Hyaluronan synthesis in mammalian cells differs from other polysaccharides in many aspects. It is elongated at the reducing end by alternate transfer of UDP-hyaluronan to the substrates UDP-GlcNac and UDP-GlcA liberating the UDP-moiety [22]. Other glucosaminoglycans grow at the non-reducing end and require a protein backbone. Hyaluronan is synthesized at plasma membranes and it was speculated that nascent chains are directly extruded into the extracellular matrix [3]. In contrast, other glucosaminoglycans are made in the Golgi. Chain initiation does not require a protein backbone as for proteoglycans, nor preformed oligosaccharides as starters, only the presence of the nucleotide sugar precursors

are sufficient to initiate new chains. During elongation the chain is retained on the membrane integrated synthase. This mechanism of synthesis operates for the synthesis in vertebrates and in gram-positive Streptococci.

Research on hyaluronan synthesis is greatly facilitated by the use of streptococci due to the ease of cultivation and the quantity of material. Hyaluronan is produced by group A and C streptococci and deposited in a capsule [35]. The hyaluronan capsule is the major virulence factor of such pathogenic streptococci [36-38]. It protects the bacteria from phagocytosis [39] and from oxygen damage [40]. Group A and C streptococci differ in their capacity to retain hyaluronan as a coat on their cell surface [41]. In group C streptococci a 56 kDa hyaluronan receptor was closely associated to the synthase. This protein had an intrinsic kinase activity that performed autophosphorylation in response to extracellular ATP. Autophosphorylation of the 56 kDa protein led to a reduction of hyaluronan binding and increased shedding of the hyaluronan capsule. Simultaneously, the synthase increased its activity to replace the lost hyaluronan chains. A large hyaluronan chain thus appears to inhibit its own elongation, when it was retained in the vicinity of the synthase. The hyaluronan synthase is a membrane protein that could be purified by a new method in active form [42].

Three genes comprising the *has* operon have been shown to encode enzymes that are involved into the synthesis of hyaluronan in Streptococci: *hasA* for the hyaluronan synthase; *hasB* for the UDP-glucose dehydrogenase, which synthesize glucuronic acid from UDP-glucose; *hasC* for the UDP-glucose phosphorylase, which forms UDP-glucose from UTP and glucose-1-phosphate. Most bacterial polysaccharides studied so far exit cells by specialized transporters. Genes encoding these transporter proteins are often found in the next vicinity of the operon that encodes enzymes utilized in the synthesis of the polysaccharide. The upstream chromosomal region of *S. pyogenes* flanking the *has* operon was partially analysed [43;44]. However, although the authors found a gene cluster containing an ABC transporter they did not inactivated the ABC transporter itself and postulated that *hasA* and *hasB* were sufficient for the capsule formation by streptococci. In another investigation irradiation inactivation was employed to calculate the size of the

capsule producing proteins [45]. From this study the authors concluded that the size excluded the participation of proteins other than the synthase itself.

The aim of our work was to further investigate the hyaluronan export from Streptococci.

Construction of a S. pyogenes mutant library

We constructed a mutant library by chromosomal integration of the plasmid pGhost9:IS*S1* harbouring an insertion element IS*S1* for gene inactivation [64]. We used the *Streptococcus pyogenes* M49 strain CS101 as a host that produced high amounts of hyaluronan and that possessed large mucoid colonies when cultured on blood agar.

Generation of hyaluronan deficient mutants

Isolation of hyaluronan deficient mutants is performed by visual appearance of the colonies. Glossy colonies produce the hyaluronan capsule while opaque colonies lack such capsules [65]. For group A streptococci this difference is only seen on blood agar plates, because whole blood contains components that repress the csrR/csrS regulators responsible for inhibition of *has* gene expression [66-68]. Among the clones with reduced mucoid appearance we found one with an unaffected synthase activity, but with reduced hyaluronan release into the medium (**Fig. 2**) and capsule production (**Fig. 3**). This mutant was selected for further characterization.

The bacterial viability of the wildtype and mutant strains was tested by vital staining. The number of dead cells after 1 and 2 hrs were 10% and 20%, respectively, and remained constant for the next 4 hrs (data not shown). This result suggested that also residual hyaluronan production of the mutant could be due to leakiness of dead bacteria.

Isolation and characterization of insertion mutants

The site of integration was determined by cloning the insertion site in *E.coli* and sequence analysis. The sequences were compared with the known sequence of

Streptococcus pyogenes M1 (strain SF370) using the data base of the University of Oklahoma Advanced Center for Genome Technology. All the genes identified in the mutant strain were at least 99% homologous to those of the sequenced M1 strain. Among mutants with inserts into the *has* gene cluster, we found a mutant with an insert into a gene that displayed strong homology to ABC transporters. The homologous gene cluster within the *Streptococcus pyogenes* M1 (strain SF370) chromosome was located in the immediate vicinity of the *has* gene cluster (**Fig. 4**). This insertion site of the vector was confirmed by PCR using primers annealing to vector sequences and genomic nucleotide sequences upstream or downstream of the *hax* locus followed by the sequencing of the PCR products. The cistron contains seven open reading frames (ORFs) that are all transcribed in the same direction in opposition to the transcription direction of the *has* gene cluster: ORF 1 encodes an unknown protein with some homology to RecA protein; ORFs 2 and 3 encode for proteins with some homology to zinc proteases; ORF 4 encodes a CDP-phosphatidylglycerol-glycerophosphate transferase; the next two ORFs, ORF 5 and 6 (where the transposon insertion was found) belong to members of an ABC transporter family (ATP binding proteins); ORF 7 encodes a putative integral membrane protein with five potential transmembrane regions; ORF 8 was found to have homology with a secretory protein SAI-B from *Staphylococcus aureus* and also to contain a putative membrane spanning region.

Rescue of hyaluronan release by streptococci by genetic complementation

A 3,8 kb chromosomal fragment comprising *haxA* to *haxD* was amplified by PCR and subcloned into pAT28 and the construct pAT28hax was obtained. When pAT28hax was transfected into the *hax* mutant, colonies displayed the mucoid phenotype on agar plates, while bacteria transfected with pAT28 only resembled the original mutant colonies. The rate of hyaluronan release of the cells transformed with pAT28hax almost reached the level of the wild type cells (**Figs. 2** and **3**). The results indicated that an ABC transporter was required for hyaluronan capsule production in intact streptococci. We have named the genes encoded in ORF 5 to ORF8 *haxA, haxB, haxC* and *haxD*.

Conclusion

In contrast to the existing paradigm, we have identified an ABC transporter that is required for hyaluronan extrusion through the cell membrane in group A *Streptococcus pyogenes*. The involved proteins are encoded at a chromosomal region immediately adjacent to the *has* genes responsible for hyaluronan synthesis. They include four ABC transporter proteins that we have named haxA, haxB, haxC and haxD. Both haxA and haxB contain an ATP binding cassette, the C-motif and a Walker-A motif that are characteristic for ABC-transporter. Insertional mutagenesis produced a mutant that was not mucoid. The mucoid phenotype was rescued by transfection with DNA encoding the four transporter genes.

To much of our surprise, we discovered that the bacterial export system showed high homology to human ABC transporters, among which the multidrug resistance transporter (MDR or ABCB) and the multidrug resistant associated proteins (MRP of ABCC) are prominent members. **Fig. 5** shows the phylogenetic relationship of Hax-A and Hax-B with human ABC transporters as calculated by the Custal method. HaxA and haxB are related to each other and to the human ABCB transporter, followed by ABCC (MPR) transporter.

Human ABC transporter are a protein family of 48 transporter that are responsible for the transport of many substrates. Many of them have a very broad substrate specificity. They are grouped in 7 subfamilies: ABCA or ABC1; ABCB or MDR; ABCC or MRP; ABCD or ALD; ABCE or OABP; ABCF or GCN20; ABCG or White (**Fig. 1**). The most important and best studies member is the P-glycoprotein that is expressed in many tissues. It exports chemostatic drugs out of tumour cells and thus makes the cells resistant towards drug treatment. However, the physiological functions of most transporters is still elusive [48].

The pharmaceutical industry spends much effort to develop improved inhibitors for these transporters [49], and therefore many inhibitors for ABC-transporters are available [50-54]. A classical inhibitor of the first generation is Verapamil that often serves as a reference compound. It is a drug that specifically inhibits the slow Na-Ca-channel and is applied under conditions of myocardial infarct and disturbances of heart rhythm (supraventricular tachycardy) that is caused by arteriosclerosis or impaired heart function. It influences Ca-influx and electromechanical coupling of

smooth muscles and can induce a rapid drop in blood pressure. It has also a long lasting dilatory effect. It lowers arterial blood pressure and protects from increased permeability in the microvascular system [55-57]. Similar effects have been described for other inhibitors of the first generation: Quinidine; Chlomipramine; Chloroquine; Quinine; Emitine; Dilthiazem; Nicardipine; Nifedipine; Bepridil; Amiloride; Cyclosporin; Rapamycin, Reserpin. In all cases the mechanisms of action is unknown. An inhibition of hyaluronan production by these drugs has not been described.

The application of Verapamil and other MDR inhibitors of the first generation in oncology was not very successful. Therefore many companies search for improved inhibitors [53;58]. A series of such drugs are currently in clinical trails, e.g. Valspodar or PSC833 from Novartis. It is a member of the immuno suppressive drugs such as Cyclosporin A and Rapamycin. The inhibitors such as valspodar and Verapamil primarily inhibit MDR (ABC-B1) transporter, but also act on MRP transporter [54]. Their effects on ABCA transporters are rather low. DIDS (4,4 -diisothiocyanatostilbene-2,2 -disulfonic acid) is a specific inhibitor of the ABC-A1 transporter and it does not inhibit the ABCB1 transporter [60]. Another specific ABCA transport inhibitor is glyburide [52]. The ABCC (MRP) transporter are reknown as organic anion transporter [50;51;54;61;62]. It can be inhibited by the general anion inhibitor benzbromarone or by the specific ABCC1 inhibitor MK-571.

We performed a series of experiments to investigate whether ABC transporters are also responsible for the export of hyaluronan from eukaryotic cells. ABC transporter inhibitors were employed to assay for inhibition of hyaluronan production in cell culture. Because hyaluronan synthesis is also required for detachment during mitosis and growth of the human HT1080 cell line [69], we also analysed the effect on cell proliferation.

ABCB1 (MRP) inhibitors

Human skin fibroblasts were grown in cell culture in the presence of increasing concentrations of the ABCB1 inhibitors Verapamil and valspodar. The amount of hyaluronan in culture medium and the cell number were determined after 3 days.

Fig. 6 shows that both Verapamil and valspodar reduced the hyaluronan transport as well as cell growth. Valspodar reduced hyaluronan transport more efficiently than Verapamil. The effective concentrations were similar to those used for the inhibitions of multidrug resistance.

Because the inhibition of hyaluronan synthesis could also be caused indirectly through the inhibition of growth, the effect of Verapamil and valspodar was also studied on human synovial fibroblasts that were expected to produce hyaluronan also under non-proliferating conditions. **Fig. 7** shows that Verapamil inhibited growth as well as hyaluronan production. In contrast, valspodar only inhibited hyaluronan production, but not cell proliferation.

Since the effect of Verapamil and valspodar could also be due to toxic effects on cellular metabolism, their influence on the hyaluronan transport in isolated membranes from human skin fibroblasts was analysed. **Fig. 8** shows that both inhibitors blocked the hyaluronan transport in a concentration dependent manner in membranes of human skin fibroblasts.

Furthermore, we used a monoclonal antibody against P-glycoprotein (C219 from Calbiochem) [70] in order to verify the participation of the MDR transporter in hyaluronan export by another method. Membranes from this cell line were incubated with and without the antibody and then assayed for hyaluronan transport. The antibody decreased the hyaluronan transport activity by 20%.

The above results suggested that the ABCB1 (MRP) transporter was involved in hyaluronan export. This finding was confirmed by comparing the effect of Verapamil on hyaluronan transport in the human colon carcinoma cell lines HT29 and HT29-mdr which differ only in the expression of the multidrug resistance protein P-glycoprotein [71;72]. **Fig. 9** shows that increasing concentrations of Verapamil more efficiently blocked hyaluronan transport in HT29 than in HT29-mdr.

Furthermore, Verapamil was tested for its effect on the hyaluronan transport activity in isolated membranes from HT29 und HT29-mdr. **Fig. 10** shows that Verapamil does not inhibit the transport in membranes from the drug resistant cell line. In the

sensitive cell line, inhibition is not complete. It is therefore possible that further transporters are involved in hyaluronan export. This hypothesis was investigated by the following experiments.

ABCA inhibitors

DIDS (4,4 -diisothiocyanatostilbene-2,2 -disulfonic acid) is a specific inhibitor of the ABC-A transporter and it does not inhibit the ABCB transporters [60]. Its effect was assayed on membranes from a human fibroblast cell line. It reduced the hyaluronan transport activity by 70% .

Another specific ABCA transport inhibitor is glyburide [52]. Its efficiency was compared with valspodar for the hyaluronan production in a human fibroblast cell line. **Fig. 11** shows that glyburide partially inhibited hyaluronan production, but not as efficient as valspodar. This experiment also indicated that the inhibitory effect of valspodar on the hyaluronan production in intact cells was stronger than on the hyaluronan transport in membranes.

ABC-C (MRP)

MRP transporter can be inhibited by benzbromarone or MK-571. These drugs were analysed for their effects on the hyaluronan transport activity in membranes from human fibroblasts and compared with valspodar. **Fig. 12** shows that benzbromarone almost completely inhibited the hyaluronan synthase activity, whereas valspodar and MK-571 were less efficient.

The results described above lead to the conclusion that more than one transporter could be involved in hyaluronan secretion from human cells and may function simultaneously in a given cell: ABC-B (MDR) transporter, ABCA transporter, and ABCC (MRP) transporter. Accordingly, in a preferred embodiment, said human ABC-transporter(s) is(are) a member of the human ABC-B (MDR)-subfamily, the ABC-Asubfamily and/or the human ABC-C (MRP)-subfamily.

The hypothesis of multiple transporters for hyaluronan may appear surprising at first glance; however, comparing the streptococcal proteins haxA and haxB with their

human counterpart also indicated that they have great homology to two different human ABC transporters ABCB and ABCC, respectively.

These results demonstrate for the first time that ABC transporter inhibitors are able to inhibit hyaluronan secretion as mediated by ABC-transporter(s) in eukaryotic cells. Many of such inhibitors have already been used and tested as drugs to treat human cancer patients (described herein elsewhere). But there exists no report on inhibition of hyaluronan production. Overproduction of hyaluronan is, however, a central problem in many diseases, in particular in ischemic or inflammatory edema or in arthritis. Also many human tumors are characterized by an overproduction of hyaluronan such as melanoma [89], mesothelioma [117] or colon carcinoma [118]. Because hyaluronan production is corellated with cell proliferation [86], inhibition of hyaluronan transport will also reduce tumor growth. Overproduction of hyaluronan is also the cause of lump formation after contusion or insect bites, therefore is will be possible to inhibit swelling by the inhibitors of hyaluronan transport. In fact, most injuries are followed by inflammation and hyaluronan overproduction that may lead to severe health problems such as organ transplantation and tissue rejection, hyaluronan production after a heart infarct, alveolitis, pancreatitis, pulmonary or hepatic fibrosis, radiation induced inflammation, Crohn's disease, myocarditis, scleroderma, psoriasis, sarcoidosis [119-135]. In particular, the unbalanced hyaluronan and proteoglycan synthesis by chondrocytes in arthritis appears to be the first biochemical event that eventually leads to complete joint destruction.

Thus, the present invention relates to the use of at least one inhibitor of at least one ABC-transporter capable of transporting hyaluronan across a lipid bilayer, for the preparation of a pharmaceutical composition for the treatment of arthritis.

It must be noted that as used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "a reagent" includes one or more of such different reagents, and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

The term "inhibitor" defines in the context of the present invention a compound or a plurality of compounds which interact(s) with one or more ABC-transporter(s) such that the hyaluronan transport mediated by such ABC-transporter(s) is reduced. It is envisaged that the inhibitors of the present invention are capable to reduce or abolish the transport of hyaluronan mediated by one or more ABC-transporter(s) across a lipid bilayer in a cell/subject in a way which is sufficient to reduce the hyaluronan-transport to at least about the same level as compared to a normal/natural state of a comparable control-cell/subject. The meaning of the "comparable control-cell/subject" is further defined herein below. The term „plurality of compounds" is to be understood as a plurality of substances which may or may not be identical. The plurality of compounds may preferably act additively or synergistically. Said compound or plurality of compounds may be chemically synthesized or microbiologically produced and/or comprised in, for example, samples, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compound(s) may be known in the art but hitherto not known to be capable of reducing the transport of hyaluronan mediated by at least one ABC-transporter.

The interaction of the inhibitor with one or more ABC-transporter(s) such that the hyaluronan transport mediated by such ABC-transporter(s) is reduced can, in accordance with this invention e.g. be effected by a reduction of the amount of the ABC-transporter(s) in cells, in tissues comprising said cells or subjects comprising said tissues or cells (for example by antisense oligonucleotides, iRNA or siRNA which specifically bind to the nucleotides sequences encoding said ABC-transporter(s) or by ribozmes which specifically degrade polynucleotides which encode ABC-transporter(s)); by blocking the binding site of the ABC-transporter(s) for hyaluronan; by competitive or allosteric inhibition of the hyaluronan transport mediated by the ABC-transporter(s) or by otherwise reducing or preventing the transport of hyaluronan mediated by one or more of the ABC-transporter(s), for example by directing antibodies and/or aptamers to such ABC-transporter(s) as defined herein and thereby reducing or preventing the hyaluronan transport mediated by said ABC-transporter(s). Thus, an example of an inhibitor of this

invention is an antibody, preferably an antibody the binding of which interferes with the transport of hyaluronan mediated by the ABC-transporters of this invention; an antisense construct, iRNA, siRNA or ribozyme constructs directed against a transcript or the coding nucleotide sequence of the ABC-transporter(s) of the invention; nucleotide sequences encoding such constructs and compounds which inhibit the transport of hyaluronan mediated by the ABC-transporter(s) as defined herein, for example by blocking or disrupting the binding site of the ABC-transporter(s) for hyaluronan or by allosteric or competitive inhibition of the hyaluronan transport mediated by the ABC-transporter(s) as defined herein.

Such inhibitors as mentioned herein are explained and discussed in more detail throughout the specification. Furthermore, the present invention provides numerous screening methods, test-systems and assays which allow the skilled person to screen for inhibitors as defined herein and to test the effect/effectiveness of such inhibitors. Such test systems are explained in great detail e.g. in the appended examples.

The term "reduced" or "reducing" as used herein defines the reduction of the hyaluronan transport across a lipid bilayer, preferably to at least about the same level as compared to a normal/natural state of a comparable control-cell/subject. Accordingly, it is understood that the reduction mediated by the inhibitor aims at "normalizing" the transport activity of ABC-transporter(s) of hyaluronan across a lipid bilayer, which does, however, not exclude that the inhibitor as defined herein might also reduce the hyaluronan transport across a lipid bilayer to a lower level as compared to a normal/natural state of a comparable control-cell/subject. It is also envisaged that the inhibitor totally abolishes the transport of hyaluronan when compared to a normal/natural state of a comparable control-cell/subject. The term "normal/natural state of a comparable control-cell/subject" means the transport-rate of hyaluronan as mediated by one or more ABC-transporter(s) in a control-cell which is preferably of the same nature as the test-cell (e.g. both cell are chondrocytes) but which is derived from a different source. "A different source" includes e.g. a cell/tissue sample obtained from a healthy subject which does not suffer from arthritis or a cell/tissue sample obtained from a distinct joint of the same subject

wherein said different joint appears to be free from arthritis-associated symptoms like the characteristic destruction of the cartilage (at least histologically). Assays and histological methods to classify arthritis and cartilage destruction associated with arthritis are well-known to the skilled person and in addition outlined in the appended examples. However, even in cases where the inhibitor will not reduce the hyaluronan-transport across a lipid-bilayer to the normal/natural state of a comparable control-cell/subject but actually reduces the hyaluronan transport when compared to the transport rate before the addition of said inhibitor, it will be appreciated that said inhibitor has a beneficial effect on the cell/tissue/subject in question.

For medical treatment it is preferable to use inhibitors that act in a reversible manner and do not block biochemical processes completely, because such drugs can be applied in a dosage that complies with the desired effect. This property is fulfilled by the described drugs.

Accordingly, it is envisaged that the inhibitor of the invention at least reduces the hyaluronan synthesis rate as mediated by an ABC-transporter to 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or even 100% when compared to synthesis rate that is achieved without the addition of said inhibitor. One specific screening assay for the hyaluronan transporter is based on the extrusion of labelled hyaluronan oligosaccharides from intact cells in monolayer culture. Said assay is further explained herein below as well as in the appended examples (Example 8). In such cases it is sufficient to analyse the effect of the inhibitor e.g. on a cell comprising one or more ABC-transporter(s), i.e. one compares the hyaluronan-transport before and after the addition of the inhibitor and thereby identifies inhibitors which reduce the transport-rate of hyaluronan across a lipid bilayer. Thus, inhibitors which exert a reduction of the hyaluronan transport across a lipid bilayer are within the scope of this invention as long as they exert a beneficial effect which means e.g. a reduction of the overall transport of hyaluronan across a lipid-bilayer; reduction of the destruction of cartilage; maintenance of the actual state of destruction of cartilage; prevention of a further destruction of the cartilage etc. restoring the healthy condition after an injury or damage. There are a number of techniques that enable the assessment of efficiency. These techniques can be

grouped into "process" measures and "outcome" measures. Products of both synthesis and degradation of cartilage can be assayed in the synovial fluid. Joint physiology can be imaged using techniques such as scintigraphy, which reflect regional blood flow and bone activity, and MRI, which will reveal the water content of the tissue. The outcome measures can be divided into four main categories - anatomical change (plane radiograph, other images), pain and stiffness, joint function (range of movement, stability), disability [90].

The reduction will also depend on the dosage and on the way of administration of the inhibitor. The dosage regimen utilizing the inhibitor of the present invention is therefore selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; and the particular compound employed. It will be acknowledged that an ordinarily skilled physician or veterinarian can easily determine and prescribe the effective amount of the compound required to prevent, counter or arrest the progress of the condition. Test-systems which are suitable for such purposes, i.e. which allow to measure the effect of an inhibitor on the hyaluronan-transport are described herein.

It is preferred that for the ABC-transporter(s) as described herein, the inhibitors of the invention have an IC50 between about 10 nanomolar and about 300 micromolar.

The term "capable of transporting hyaluronan across a lipid bilayer" means that the ABC-transporter(s) as described herein is(are) able to transport hyaluronan as defined herein from one compartment to another compartment, wherein both compartments are separated by a lipid bilayer. The term "lipid bilayer" is well-known to the skilled person [91] and denotes e.g. biological membranes or liposomes. Assay and test-systems which allow the determination of hyaluronan-transport across a lipid bilayer are explained in the appended examples in great detail. It will be understood that the term "capable of transporting hyaluronan across a lipid bilayer" defines in the context of cells or tissues comprising said cells, the transport of hyaluronan to the exterior of the cell (e.g. the extracellular milieu).

It has to be understood that in the context of the present invention, the term "at least one inhibitor" or "at least one ABC-transporter(s)" comprises at least one, at least two, at least three, at least four, at least five, at least six ...etc. inhibitor(s)/ABC-transporter(s). In some cases it will be sufficient if the inhibitor interacts with, and thereby inhibits only one ABC-transporter which is present in a cell/tissue in question and which is able to transport hyaluronan across a lipid bilayer, although other ABC-transporter(s) are present (regardless whether these additional ABC-transporter(s) are able to transport hyaluronan or not). It will be understood that in such particular cases, the inhibition of one ABC-transporter is sufficient to reduce the transport of hyaluronan across a lipid bilayer to such an extent that the overall transport of said cell is reduced to the same/ or below the level as compared to a normal/natural state of a comparable control-cell/subject or at least to such an extent which exerts a beneficial effect to the cell/tissue/subject (beneficial effect can be e.g. reduction of the overall transport of hyaluronan across a lipid-bilayer; reduction of the destruction of cartilage; maintenance of the actual state of destruction of cartilage; prevention of a further destruction of the cartilage etc.). In other cases, it is envisaged that the inhibitor interacts with and thereby inhibits more than one ABC-transporter which is present in the cell/tissue/subject in question and which are able to transport hyaluronan across a lipid bilayer. In such cases it might be desirable to use e.g. at least two inhibitors (or more) which might act additive or synergistically. Said two or more inhibitors will be selected in accordance with the specific conditions which are to be treated, i.e. it might be desirable to combine two or more inhibitors which are distinct with respect to the hydrophobicity, molecular mass, antigenic characteristics, route of administration, half-life in blood or serum etc. Alternatively, the inhibitor itself can be constructed to encompass two or more different entities which are able to inhibit the transport of hyaluronan across a lipid bilayer (by way of inhibiting one or more ABC-transporter(s)) and which are linked for example via peptide bonds or other suitable linkers like, e.g. chemical bonds. Suitable methods/linkers to connect two or more compounds are well-known in the art. In such a particular case one inhibitor (which actually consists e.g. of two different inhibiting entities) is e.g. able to inhibit e.g. two or more different ABC-transporter(s) which are able to transport hyaluronan across a lipid bilayer. It will be

understood that the number of inhibitors and or the number of ABC-transporter(s) which are to be inhibited by said inhibitors will be selected on a case to case basis in order to provide a suitable treatment for the cell/tissue/subject. In this context, "suitable" means that the treatment with the respective inhibitor(s) of the invention exerts a beneficial effect, e.g. it prevents, counters or arrests the progress of the condition (e.g. reduction of the overall transport of hyaluronan across a lipid-bilayer; reduction of the destruction of cartilage; maintenance of the actual state of destruction of cartilage; prevention of a further destruction of the cartilage etc.). The number of inhibitors and or the number of ABC-transporter(s) which are to be inhibited by said inhibitors by utilizing the inhibitors of the present invention for treating or preventing a disease which is associated with arthritis (described elsewhere in this specification), is selected in accordance with a variety of factors including type, species, age, weight, sex and so on.

In a preferred embodiment of the use or the methods of the present invention said inhibitor(s) specifically reduce(s) the transport of hyaluronan across a lipid bilayer mediated by at least one of said ABC-transporter(s). The term "specifically reduce(s)" used in accordance with the present invention means that the inhibitor specifically causes a reduction of the transport of hyaluronan as mediated by ABC-transporter(s) but has no or essentially has no significant effect on other cellular proteins or enzymes. It will be understood that the inhibitor(s) of the present invention may also have an influence on the hyaluronan-synthase, e.g. the activity of the hyaluronan-synthase is reduced or abolished by the respective inhibitor(s), although such inhibitor(s) are not particularly preferred in the context of the present invention. Alternatively, the inhibitor has no or essentially no influence on the hyaluronan-synthase, which means that the hyaluronan-synthase activity is not reduced or only reduced by 5%, 10%, 15%, 20%, 25%, 30%, 35% or 40%. The specificity of the inhibitor, e.g. with respect to the hyaluronan-synthase or the ABC-transporter(s) can be measured e.g. by expression of the synthase or ABC-transporter in a suitable cell which, as such, is deficient of the corresponding hyaluronan-synthase or ABC-transporter (e.g. a bacterial cell). The inhibitors can also be discriminated by virtue of their binding to the respective proteins. Synthase inhibitors such as peroidate oxidized nucleotide sugars will bind to the synthase and

inhibitors of hyaluronan transport will bind to ABC-transporter(s) [24,25]. Methods have been described that assay the binding of inhibitors to ABC transporters [92;93]. Indirectly acting inhibitors that change the functional activity of the synthase or the transporter will bind to neither one, but to intracellular signalling factors. One specific screening assay for the hyaluronan transport as mediated by the ABC-transporter(s) is based on the extrusion of labeled hyaluronan oligosaccharides from intact cells in monolayer culture. For this assay the labeled oligosaccharides have to be introduced into the cytosol of cells. Because they will normally not transverse the plasma membranes, they are introduced by osmotic lysis of pinocytotic vesicles according to a method that has already successfully been applied for the introduction of periodate oxidized nucleotide sugars [25]. Hyaluronan oligosaccharides are prepared from commercially available hyaluronan by digestion with hyaluronidase and sized fractionation by gel filtration as described [102]. Appropriate oligosaccharide fractions having a lenght between 2 and 50 disaccharide units are labeled by incorporation of a biotin, radioactivity, or a fluorescent probe. These methods are routine published procedures [87,99-101,103]. The cells are seeded into multiwell microtiter plates to a density of at least 4x104 cells/cm^2. When the cells are attached to the plastic surface after a few hours, they are washed with phosphate buffered saline and incubated with the labeled hyaluronan dissolved in medium for osmotic lysis of pinocytotic vesicles (growth medium such as Dulbeccos medium containing 1 M sucrose, 50% poly(ethylene glycol)-1000) for at least 5 min up to several hours at 37°C. During this time the cells will pinocytose this hyperosmotic medium and the labeled hyaluronan. The above medium is substituted by a mixture of Dulbeccos medium and water (3:2) for 2 min. This causes the intracellular pinocytotic vesicles to lyse and to liberate the contents into the cytosol without damaging the cells. The cells can be subjected to this incubation sequence several times. The cells are washed thoroughly several times with phosphate buffered saline or growth medium to remove extracellular labeled hyaluronan and are then ready for the assay. They are incubated in growth medium containing the compound to be tested in different concentrations for several hours. During this time the labeled hyaluronan will be transported back into the medium. The amount of labeled hyaluronan

oligosaccharide in the medium can be determined by a biotin-related assay, by radioactivity or by fluorescence intensity.

It will be understood that the above method is only an exemplary method. Other methods which are also within the gist of the present invention are described herein below as well as in the appended examples.

In a particularly preferred embodiment, said inhibitor specifically reduces only such ABC-transporter(s) which are able to transport hyaluronan across a lipid-bilayer but does not or essentially does not inhibit other ABC-transporter(s), which are not able to transport hyaluronan across a lipid bilayer. Test-systems and methods for measuring the specific transport of hyaluronan across a lipid bilayer as well as methods for analyzing the transport of hyaluronan mediated by an ABC-transporter are well known to the skilled person and also provided in the appended examples. For example ABC transporters can specifically be identified as described above with the MDR transporter in HT29 and HT29-mdr cells. The gene for the transporter is transfected and the protein is expressed in a given cell line and the hyaluronan transport activities are compared (of the parent and the transfected cells) towards the responsiveness of the inhibitors.
Only those inhibitors that inhibit the ABC-transporter(s) of interest (i.e. ABC-transporter(s) which are able to transport hyaluronan across a lipid bilayer) but do not or do not essentially bind to any of the other ABC-transporter(s) which are preferably expressed by the same cell or tissue are considered to be specific for the ABC-transporter(s) of interest (i.e. ABC-transporter(s) which are able to transport hyaluronan across a lipid-bilayer).

In another embodiment of the uses of the present invention, said ABC-transporter(s) is(are) a mammalian ABC-transporter(s).

In a preferred embodiment said mammalian ABC-transporter(s) is(are) a human ABC-transporter(s).

The ABC superfamily is one of the largest families of proteins. The most recent annotation of the human genome sequence revealed 48 genes for ABC proteins. The ABC-transporters were grouped into seven sub-classes, ranging from ABCA to ABCG [see e.g.: http://nutrigene.4t.com/humanabc.htm] based on genomic organization, order of domains and sequence homology. The known 48 human ATP-transporter(s) including their Accession numbers are depicted below as well as in **Figure 1**.

48 Human ATP-Binding Cassette Transporters

Name	ABC1	MDR	MRP	ALD	OABP	GCN20	White
Subfamily	ABCA	ABCB	ABCC	ABCD	ABCE	ABCF	ABCG
Members	12	11	12	4	1	3	5 (+1?)
Some Inhibitors	DIDS Glyburide	Verapamil Valspodar Nicardipin Nimodipin	MK-571 Benzbromaron				

Symbols Access						
ABCA1: NM005502	ABCB1 NM000927	ABCC1 NM 004996	ABCD1 NM 000033	ABCE1 NM 002940	ABCF1 NM 001090	ABCG1 NM 004915
ABCA2: NM001606	ABCB2 NM000593	ABCC2 NM 000392	ABCD2 NM 005164		ABCF2 NM 005692	ABCG2 NM 004827
ABCA3 NM001089	ABCB3 NM000544	ABCC3 NM 003786	ABCD3 NM 002858		ABCF3 AK002060	ABCG3
ABCA4 NM000350	ABCB4 NM000443	ABCC4 NM 005845	ABCD4 NM 005050			ABCG4 NM 022169
ABCA5 NM018672	ABCB5 U66692	ABCC5 NM 005688				ABCG5 NM 022436
ABCA6 NM080284	ABCB6 NM 005689	ABCC6 NM 001171				ABCG8 NM 022437
ABCA7 NM019112	ABCB7 NM004299	ABCC7 NM 000492				
ABCA8 NM007168	ABCB8 NM 007188	ABCC8 NM 000352				
ABCA9 NM080283	ABCB9 NM 019624	ABCC9 NM 005691				
ABCA10 NM080282	ABCB10 NM 012089	ABCC10 XM052745				
ABCA12 NM015657	ABCB11 NM 003742	ABCC11 NM 033151				
ABCA13		ABCC12 NM 033226				

It will be understood that the sequences of the indicated ABC-transporter(s) may also deviate from the above indicated accession sequences. It is therefore envisaged that also ABC-transporter(s) which share a homology (level of nucleic acid sequence) of at least 70%, preferably 80%, more preferred 90% and even more preferred above 95% identity with the above outlined ABC-transporter(s) are within the scope of the present invention. Other ABC-transporter(s), e.g. from other species or from the same species but from different sources can easily be identified, e.g. by sequence analysis using known programs like BLAST or by screening

methods based on hybridizing ABC-transporter probes (nucleic acid probes for the identification of species homologues and the like). Such methods are well-known to the skilled person.

It is also envisaged that the term "ABC transporter" includes a superfamily of genes found in many organisms including humans (Higgins CF, "ABC transporters: from microorganisms to man", Annu Rev Cell Biol 8:67-113 (1992)) characterized by a highly conserved region known as the ATP binding cassette (Hyde et al., "Structural model ofATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport", Nature 346:362-365 (1990); Mimura et al., "Structural model of the nucleotide binding conserved component of periplasmic permeases", Proc Natl Acad SciUSA 88:84-88 (1991)). These characteristic features provide a means of identifying unknown members of the ABC transporter superfamily using various database searching techniques, e.g. BLAST (Altschul et al., "Basic local alignment search tool," JMol Biol 215:403-410 (1990)). Using the N-terminal ATP-binding domain of MDR1 as a conserved region of the superfamily of ABC transporters, this strategy has been utilized to identify a large family of ESTs corresponding to putative ABC transporters (Allikmets et al., "Characterization of the human ABC superfamily: isolation and mapping of 21 new genes using the Expressed Sequence Tags database", Human MolGenetics 5:1649-1655 (1996)). Accordingly, such new and not yet identified ABC-transporters are also within the gist of the present invention. Suitable methods for isolating/screening such new ABC-tranporter(s) and for isolating homologues of such ABC-transporter(s) (i.e. homologues from other species like other mammals or the like) are well known to the skilled person (see for example; Sambrook et al, loc. cit). Such homologues as well as orthologs and analogs are for example detailed in WO9848784 which is also incorporated herein by reference.

In a preferred embodiment said human ABC-transporter(s) is(are) a member of the human ABCB (MDR)-subfamily, the ABCA subfamily and/or the human ABC-C (MRP)-subfamily.

Chondrocytes represent only 5% of the tissue. They are responsible for synthesizing and controlling the matrix. The biosynthesis of hyaluronan and proteoglycans have

different mechanisms and occur in different compartments [2;3]: Proteoglycans are synthesized in the Golgi and exocytosed by vesicles. Hyaluronan is polymerized at the inner side of plasma membranes and exported by ABC-transporters. Both components aggregate in the extracellular matrix. In osteoarthritic cartilage the matrix appears more swollen and amorphous [4].

Chondrocytes alter the rates of syntheses in osteoarthritic cartilage. Hyaluronan production is increased compared to proteoglycans [5], the CD44-receptor is down regulated and enhanced synthesis of proteases and collagenases causes dissolution of collagen fibrils. Finally chondrocytes go into apoptosis. These events are triggered by cellular mediators, such as interleukin-1, and can also be observed in cell cultures. Limited interleukin-1 treatment increases proteoglycan synthesis and its concentrations returns to normal without significant over production. Thus chondrocytes appear to have a rudimentary memory. They sensor matrix loss and respond with repair. The sensors could be the CD44 receptors, because they can trigger an intracellular reaction cascade upon hyaluronan binding at the cell surface [6-10]. Osteoarthritic cartilage has lost this memory [11]. In addition to the above-described trigger via cellular mediators, almost any disturbance of the cellular homeostasis results in stimulation of hyaluronan production.

The rates of synthesis and degradation are similar in healthy cartilage. Hyaluronan can be endocytosed by CD44 and proteoglycans leave the cartilage by diffusion [12]. The well synchronized equilibrium between catabolic and anabolic processes is disturbed by cytokines and proteases. It has been demonstrated in cell cultures that interleukin-1 stimulates hyaluronan and protease synthesis and inhibits aggrecan and CD44 synthesis [9;10;13-15]. Increased hyaluronan synthesis is the primary process and occurs before the stimulation of protease synthesis [16;17]. For a long time it was thought that proteolytic degradation of collagen and aggrecan was responsible for cartilage dissolutions. However, the inefficiency of proteinase inhibitors converts this hope into an illusion [18].

It is known that the CD44 receptor transmits signals into the cell upon binding to oligosaccharides of hyaluronan and causes increased production of metalloproteases [19]. Therefore it appears likely that an enhanced hyaluronan concentration in osteoarthritic cartilage has a similar effect and also induces

protease production. But is also possible that overproduction hyaluronan is alone sufficient to desintegrate cartilage. The equilibrium between proteoglycans and hyaluronan is disturbed and the strongly swelling hyaluronan looses aggrecan decoration like a bottle brush without bristles. Permanent new synthesis of hyaluronan pushes intact proteoglycan aggregates away from chondrocytes to soften the cartilage matrix.

Therefore the primary aim of treatment must include a restriction of hyaluronan production in activated chondrocytes.

Accordingly, in a further embodiment of the present invention said ABC-transporter(s) is(are) comprised in a chondrocyte cell, preferably a human chondrocyte cell. Chondrosarcoma cell lines such as CRL-7891 or HTB-94 from the American Type Culture Collection are particularly suitable for in vitro investigations on hyaluronan transport, because they produce large amounts of hyaluronan binding proteoglycan in cell culture [107-109] and constitutively express P-glycoprotein [110-112]. The respective ABC-transporter(s) which is(are) comprised in such a chondrocyte cell or cell-line can be easily detected, e.g. by FACS-analysis, Northern-Blot; Western-Blot, RT-PCR and the like. The respective nucleic acid sequences encoding the ABC-transporter(s) are described elsewhere in this specification (e.g. in Figure 1). With regard to FACS-analysis and other protein-based assays, it is e.g. possible to employ specific antibodies which specifically detect the different ABC-transporter(s). Examples of such antibodies have been described elsewhere in this specification.

It is also known that human chondrocytes can express the ABCB1- (MDR-) gene product P-glycoprotein under certain conditions [94;95]. As mentioned before, there are several methods that allow the identification of ABC transporters in chondrocytes. First, specific antibodies can be used in histochemical immunofluorescence or in ELISA assays to detect the corresponding antigen. Second, the level of transcription can be measured by quantitative RT-PCR. Third, in situ hybridization with specific oligonucleotide cDNA probes. Fourth, microarray chip technology can be employed, wherein the expression pattern of the ABC-

transporter(s) in the cell is detected via a predetermined number of given ABC-transporter-sequences on said chip. All the aforementioned methods are well-established and of course well-known to the skilled artisan.

The action of the MDR inhibitor valspodar on hyaluronan synthesis was first investigated in a temperature sensitive human chondrocyte cell line that proliferates at 32°C and differentiates to chondrocytes at 39°C. These chondrocytes respond to interleukin in a similar way as chondrocytes obtained brom biopsy [73]. The chondrocytes were cultured for 5 days at 39°C in alginate beads and then incubated in the presence of increasing concentrations of valspodar. After 3 days the media were withdrawn and the beads were solubilized with citrate buffer. The chondrocytes were harvested by centrifugation. The concentrations of hyaluronan and proteoglycans were determined in the media, the alginate and the cells. [35S]Sulfate incorporation into proteoglycans was determined in a parallel series of experiments during the last 24 hours of incubation. The hyaluronan concentration was determined by an ELISA assay [74]. The proteoglycan concentration was measured by a colour reaction [75], and sulfate incorporation into proteoglycans was determined by radioactivity [76].

Fig. 13 shows that increasing valspodar concentrations cause a drop in the hyaluronan concentration in interleukin induced on chondrocytes and in the alginate beads to comparable levels of interleukin free cultures. The dramatic increase of hyaluronan production in alginate verifies previous results [14]. In alginate the hyaluronan does not disappear completely. This could be due to the fact that residual concentrations of hyaluronan remained in alginate from the preceding incubation that were not washed out by the media change.

Figure 14 shows that increasing valspodar concentrations do not cause any change in the proteoglycan synthesis rate and its concentration in alginate. The dramatic decrease of proteoglycan synthesis verifies previous results [14]. However, valspodar causes a drop in the proteoglycan concentration on the cells. This result can be explained by the assumption that the limited hyaluronan concentration was not sufficient to retain proteoglycans.

These results show that valspodar selectively inhibits hyaluronan synthesis in humane chondrocytes and does not influence proteoglycan synthesis. Many other ABC transporter inhibitors are acting in a similar way as valspodar and have the same target [53]. Therefore they will exert a similar inhibitory effect on hyaluronan production. Several inhibitors have been tested to prove this hypothesis (the respective results are described herein below).

Accordingly, in a prefered embodiment of the present invention said inhibitor(s) is(are) selected from the group consisting of:

(a) an inhibitor of a member of the ABCB (MDR)-subfamily selected from Verapamil, Valspodar (PSC833), Elacridar (GF-120918), Bericodar (VX-710), Tariquidar (XR-9576), XR-9051, S-9788, LY-335979, MS 209, , R101933; OC-144-093; Quinidine, Chloripramine, Nicardipine, Nifedipine, Amlodipine, Felodipine, Manidipine, Flunarizine, Nimodipine, Pimozide, Lomerizine, Bepridil, Amiloride, Almitrine, Amiodarone, Imipramine, Clomiphene, Tamoxifen, Toremifene, Ketocanazole, Terfenadine, Chloroquine, Mepacrin, Diltiazem, Niguldipine, Prenylamine, Gallopamil, Tiapamil, Dex-Verapamil, Dipyridamole, Pimozide, Haloperidol, Chlorpromazine, Trifluoperazine, Fluphenazine, Reserpin, Clopenthixol, Flupentixol, N-acetyldaunorubicin, Vindoline, N276-14, N276-17, B9309-068, BIBW-22, Carvedilol, Clofazimine, Ketoconazole, Lovastatin, N-Norgallopamil, Simvastatin, Troleandomycin, Vinblastin, Itraconazole, Econazole, Oligomycine, Cyclosporin and Rapamycin ; and/or

(b) an inhibitor of a member of the ABCA subfamily selected from Glyburide, DIDS (4,4-diisothiocyanatostilbene-2,2-disulfonic acid), Bumetanide, Furosemide, Sulfobromophthalein, Diphenylamine-2-carboxylic acid and Flufenamic acid; and/or

(c) an inhibitor of a member of the human ABC-C (MRP)-subfamily selected from MK-571, Benzbromaron, PAK-104P, Probenecid, Sulfinpyrazone, Indomethacin, Merthiolate and Ethacrynic acid; and/or

(d) (an) antibody(ies) or functional fragments thereof which is(are) specifically recognizing one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer; and/or

(e) (an) antisense oligomere(s), iRNA and/or siRNA directed against one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer; and/or

(f) (an) aptamer(s) directed against one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer.

The meaning and scope of the terms N276-14, N276-17, B9309-068, is well-described in the following references, which are therefore incorporated herein by reference. The compound B9309-068 (3,4-dihydro-2,7,dimethyl-3-[2-(4-morpholinyl)ethyl]-5-(3-nitrophenyl)-4-oxo-6-[1-oxo-1]-(4.4-diphenyl-1-piperidinyl)undecyl)-p-yrido[2,3-d]pyrimidine-dihydrochloride) is described on pages 157-168 of Beck,J.F., Buchholz,F., Ulrich,W.R., Boer,R., Sanders,K.H., Niethammer,D., & Gekeler,V. (1998) Rhodamine 123 efflux modulation in the presence of low or high serum from CD56+ hematopoietic cells or CD34+ leukemic blasts by B9309-068, a newly designed pyridine derivative. Cancer Lett., 129, 157-163. The compounds terms N276-14, N276-17 are described on pages 123-132 of Naito,S., Koike,K., Ono,M., Machida,T., Tasaka,S., Kiue,A., Koga,H., & Kumazawa,J. Development of novel reversal agents, imidazothiazole derivatives, targeting MDR1- and MRP-mediated multidrug resistance. Oncol.Res. 10, 123-132. 1996.
The term/compound LY-335979 is for example described in (Dantzig et al., (1996) CancerResearch 56:4171-4179) as well as in WO9917757.

Further references for some specific inhibitors which are also within the scope of the present invention are exemplified below:
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As mentioned before, several inhibitors of ABC-transporter(s) have already been used in the past in the context of a reduction of the so-called multi-drug-resistance in tumors. The known multidrug resistance inhibitors have particularly been designed and optimized to block the export of chemostatic drugs in human tumours by ABC transporters. But these drugs are not the physiological substrates for the transporters. Our work demonstrated for the first time that hyaluronan is a physiological substrate and that its production can be inhibited by the known inhibitors. It is therefore a great advantage that these inhibitors and the knowledge that has accumulated with the use of multidrug resistance inhibitors can now be utilized to inhibit hyaluronan production for the treatment of human diseases (as described herein). Accordingly, it is envisaged that the use of such inhibitors is encompassed by the present invention (this also relates to such inhibitors which are not mentioned *expressis verbis* herein – however, the skilled artisan has access to such ABC-transporter inhibitors, e.g. by searching the internet, review articles, textbooks and the like which relate to and/or disclose ABC-transporter inhibitors). Reference may be made to the literature for sources of further appropriate ABC-transporter inhibitors to use according to the invention. Thus, it has to be understood that the inhibitor(s) of the invention are not limited to the specific inhibitor(s) mentioned herein. The following inhibitor(s) merely represent an preferred selection of suitable inhibitor(s) within the meaning of the present invention.

Furthermore, it has to be understood that the specific inhibitor(s) specified for example herein, can be further modified to achieve (i) modified site of action, spectrum of activity, organ specificity, and/or (ii) improved potency, and/or (iii) decreased toxicity (improved therapeutic index), and/or (iv) decreased side effects, and/or (v) modified onset of therapeutic action, duration of effect, and/or (vi) modified pharmakinetic parameters (resorption, distribution, metabolism and excretion), and/or (vii) modified physico-chemical parameters (solubility,

hygroscopicity, color, taste, odor, stability, state), and/or (viii) improved general specificity, organ/tissue specificity, and/or (ix) optimized application form and route by (i) esterification of carboxyl groups, or (ii) esterification of hydroxyl groups with carbon acids, or (iii) esterification of hydroxyl groups to, e.g. phosphates, pyrophosphates or sulfates or hemi succinates, or (iv) formation of pharmaceutically acceptable salts, or (v) formation of pharmaceutically acceptable complexes, or (vi) synthesis of pharmacologically active polymers, or (vii) introduction of hydrophylic moieties, or (viii) introduction/exchange of substituents on aromates or side chains, change of substituent pattern, or (ix) modification by introduction of isosteric or bioisosteric moieties, or (x) synthesis of homologous compounds, or (xi) introduction of branched side chains, or (xii) conversion of alkyl substituents to cyclic analogues, or (xiii) derivatisation of hydroxyl group to ketales, acetales, or (xiv) N-acetylation to amides, phenylcarbamates, or (xv) synthesis of Mannich bases, imines, or (xvi) transformation of ketones or aldehydes to Schiff's bases, oximes, acetales, ketales, enolesters, oxazolidines, thiozolidines or combinations thereof.

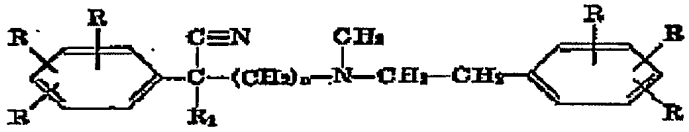
Several MDR modulators of the third generation have recently been developed using sturcture-activity relationships and combinatorial chemistry approaches targeted against specific MDR mechanisms. These agents exhibit effective inhibiting concentrations in the nanomolar range. These agents are better tolerated.

In a preferred embodiment said inhibitor is selected from the group consisting of Verapamil, Valspodar (PSC833), Elacridar (GF-120918), Bericodar (VX-710), Tariquidar (XR-9576), XR-9051, S-9788, LY-335979, MS 209, Laniquidar (R101933); OC-144-093; BIBW-22. Said compounds are, for example, described in US 3,261,859 (Verapamil); in WO 94/07858 (Bericodar); in US 5,405,843 or EP 0 363 212 B1 (MS 209); in EP-B1 0 296 122 (Valspodar (PSC833)), in WO 98/17648 (Tariquidar (XR-9576)); in EP-B1 0 466 586 (S-9788); in US 5,756,527 (OC-144-093); in EP-A1 0 494 623 (Elacridar (GF-120918)); DE 4225353 (BIBW-22)

The following compounds illustrate inhibitors which are to be used as inhibitors of the present invention.

Verapamil; US 3,261,859:

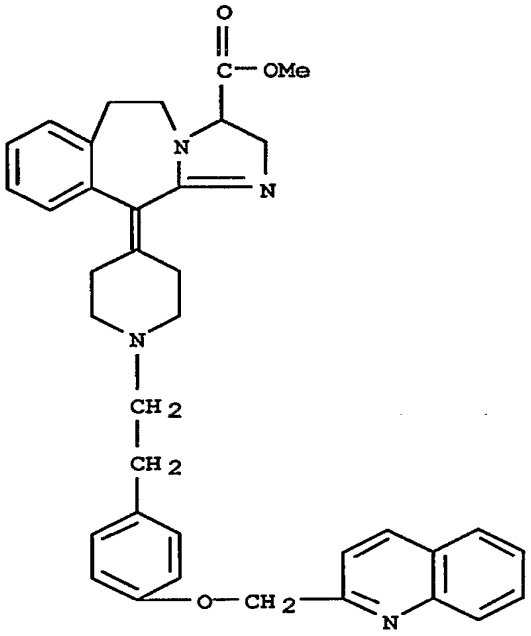
1. The compound of the formula



in which
R_1 is a member selected from the group consisting of lower alkyl, cyclohexyl, and phenyl,
R is hydrogen and at least one of the following: chlorine, lower alkoxy, lower alkyl, and n is an integer from 2 to 3, inclusive,
or its pharmaceutically acceptable acid addition salts.

Further derivatives as well as methods for producing said compound are detailed in US 3,261,859 which is, therefore, included herein in its entirety by reference.

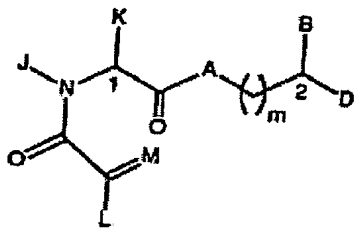
Laniquidar (R101933);



$C_{37}H_{36}N_4O_3$. Methyl 6,11-dihydro-11-[1-[2-[4-(-2-quinolylmethoxy)phenyl]ethyl]-4-piperidinylidene]-5*H*-imida-zo[2,1-*b*][3]benzazepine-3-carboxylate. CA 278798-78-0

Bericodar (VX-710); WO 94/07858

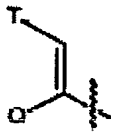
1. A compound of formula (I):



(I)

wherein A is CH_2, oxygen, NH or N-(C1-C4 alkyl);
wherein B and D are independently

(i) Ar, (C1-C10)-straight or branched alkyl, (C2-C10)-straight or branched alkenyl or alkynyl, (C5-C7)-cycloalkyl substituted (C1-C6)-straight or branched alkyl, (C2-C6)-straight or branched alkenyl or alkynyl, (C5-C7)-cycloalkenyl substituted (C1-C6)-straight or branched alkyl, (C2-C6)-straight or branched alkenyl or alkynyl, or Ar substituted (C1-C6)-straight or branched alkyl, (C2-C6)-straight or branched alkenyl or alkynyl wherein, in each case, any one of the CH_2 groups of said alkyl, alkenyl or alkynyl chains may be optionally replaced by a heteroatom selected from the group consisting of O, S, SO, SO_2, N, and NR, wherein R is selected from the group consisting of hydrogen, (C1-C4)-straight or branched alkyl, (C2-C4)-straight or branched alkenyl or alkynyl, and (C1-C4) bridging alkyl wherein a bridge is formed between the nitrogen and a carbon atom of said heteroatom-containing chain to form a ring, and wherein said ring is optionally fused to an Ar group; or

(ii) 

wherein Q is hydrogen, (C1-C6)-straight or branched alkyl or (C2-C6)-straight or branched alkenyl or alkynyl;

wherein T is Ar or substituted 5-7 membered cycloalkyl with substituents at positions 3 and 4 which are independently selected from the group consisting of oxo, hydrogen, hydroxyl, O-(C1-C4)-alkyl or O-(C2-C4)-alkenyl;

provided that at least one of B or D is independently selected from the group consisting of (C2-C10)-straight or branched alkynyl, (C5-C7)-cycloalkyl substituted (C2-C6)-straight or branched alkynyl, (C5-C7)-cycloalkenyl substituted (C2-C6)-straight or branched alkynyl, and Ar substituted (C2-C6)-straight or branched alkynyl;

wherein Ar is a carbocyclic aromatic group selected from the group consisting of phenyl, 1-naphthyl, 2-naphthyl, indenyl, azulenyl, fluorenyl, and anthracenyl; or a

heterocyclic aromatic group selected from the group consisting of 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, pyrrolyl, oxazolyl, thiazolyl, imidazolyl, pyrazolyl, 2-pyrazolinyl, pyrazolidinyl, isoxazolyl, isotiazolyl, 1,2,3-oxadiazolyl, 1,2,3-triazolyl, 1,3,4-thiadiazolyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,3,5-triazinyl, 1,3,5-trithianyl, indolizinyl, indolyl, isoindolyl, 3H-indolyl, indolinyl, benzo[b]furanyl, benzo[b]thiophenyl, 1H-indazolyl, benzimidazolyl,

benzthiazolyl, purinyl, 4H-quinolizinyl, quinolinyl, isoquinolinyl, cinnolinyl, phthalazinyl, quinazolinyl, quinoxalinyl, 1,8-naphthyridinyl, pteridinyl, carbazolyl, acridinyl, phenazinyl, phenothiazinyl, and phenoxazinyl;

wherein Ar may contain one to three substituents which are independently selected from the group consisting of hydrogen, halogen, hydroxyl, nitro, trifluoromethyl, trifluoromethoxy, (C1-C6)-straight or branched alkyl, (C2-C6)-straight or branched alkenyl, O-(C1-C4)-straight or branched alkyl, O-(C2-C4)-straight or branched alkenyl, O-benzyl, O-phenyl, 1,2-methylenedioxy, amino, carboxyl, N-(C1-C5-straight or branched alkyl or alkenyl) carboxamides, N,N-di-(C1-C5-straight or branched alkyl or C2-C5-straight or branched alkenyl)carboxamides, N-morpholinocarboxamide, N-benzylcarboxamide, N-thiomorpholinocarboxamide, N-picolinoylcarboxamide, O-X, CH_2-$(CH_2)_q$-X, O-$(CH_2)_q$-X, $(CH_2)_q$-O-X, and CH=CH-X; wherein X is 4-methoxyphenyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, pyrazyl, quinolyl, 3,5-dimethylisoxazoyl, isoxazoyl, 2-methylthiazoyl, thiazoyl, 2-thienyl, 3-thienyl, and pyrimidyl, and q is 0-2;

wherein L is either hydrogen or U; M is either oxygen or CH-U, provided that if L is hydrogen, then M is CH-U or if M is oxygen, then L is U;

wherein U is hydrogen, O-(C1-C4)-straight or branched alkyl or O-(C2-C4)straight or branched alkenyl, (C1-C6)-straight or branched alkyl or (C2-C6)-straight or branched alkenyl, (C5-C7)-cycloalkyl or (C5-C7)-cycloalkenyl substituted with (C1-C4)-straight or branched alkyl or (C2-C4)-straight or branched alkenyl, [(C1-C4)-alkyl or (C2-C4)-alkenyl]-Y or Y;

wherein Y is a carbocyclic aromatic group selected from the group consisting of phenyl, 1-naphthyl, 2-naphthyl, indenyl, azulenyl, fluorenyl, and anthracenyl; or a

heterocyclic aromatic groups as defined above;

wherein Y may contain one to three substituents which are independently selected from the group consisting of hydrogen, halogen, hydroxyl, nitro, trifluoromethyl, trifluoromethoxy, (C1-C6)-straight or branched alkyl, (C1-C6)-straight or branched alkenyl, O-(C1-C4)-straight or branched alkyl, O-(C2-C4)-straight or branched alkenyl, O-benzyl, O-phenyl, 1,2-methylenedioxy, amino, and carboxyl;

wherein J is hydrogen, (C1-C2) alkyl or benzyl; K is (C1-C4)-straight or branched alkyl, benzyl or cyclohexylmethyl, or wherein J and K may be taken together to form a 5-7 membered heterocyclic ring which may contain a heteroatom selected from the group consisting of O, S, SO and SO_2; and

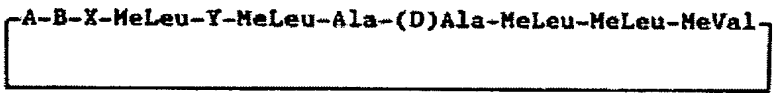
wherein m is 0-3.

Further derivatives as well as methods for producing said compound are detailed in WO 94/07858 which is, therefore, included herein in its entirety by reference.

XR-9051 (3-[(3Z, 6Z)-6 Benzylidene- 1 -methyl-2 ,5 -dioxopiperazin-3 -ylidenemethyl] -N-[4-[2-(6,7-dimethoxy- 1,2,3 ,4-tetrahydroisoquinolin-2-yl)ethyl]phenyl]benzamide hydrochloride, [AC # 57-22- 7]).

Valspodar; EP-B1 0 296 122:

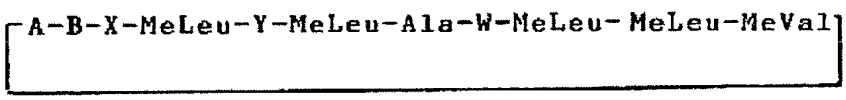
A compound (i) of formula II



(II)

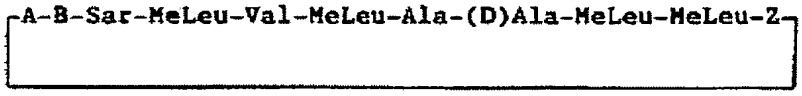
wherein
A is -3'-O-acetyl-MeBmt-
B is -αAbu-, -Thr-, -Val- or Nva-; and
when
B is -αAbu-,
X is -(D)Ala- and Y is -Val-;
when
B is -Thr- or -Val-,
X is -Sar- and Y is -Val-; or
when
B is -Nva-,
X is -Sar- and Y is -Val-, or
X is -(D)Ala- and Y is -Val-; or
wherein
A is -3'-O-acetyl-dihydro-MeBmt- or -cis-MeBmt-,

B is -αAbu-, X is -Sar- and Y is -Val-; or
ii) of formula II'



(II')

wherein
A is -3'-O-acyl-MeBmt- or -3'-O-acyl-dihydro-MeBmt- residue,
B is -αAbu-, -Thr-, -Val-, -Nva-, or the residue of a β-O-acyl-α-amino acid,
X is -Sar- or the residue of an optically active α-N-methylated α-amino acid residue having the (D)-configuration,
Y is -Val- or additionally, when B is -Nva-, -Nva-, and
W is the residue of a β-hydroxy- or β-O-acyl-α-amino acid having the (D)-configuration; or
iii) wherein the residue at the position 1-position is an -8'-C_{1-6}alkoxy-cis-MeBmt-or -dihydro-MeBmt- or -3'-O-acyl-8'-C_{1-6}alkoxy-cis-MeBmt- or -dihydro-MeBmt- residue; a -3'-O-acyl-cis-MeBmt-residue; a -7'-desmethyl-7'-hydrocarbyl- -MeBmt- or -cis-MeBmt- or -3'-O-acyl-7'-desmethyl-7'-hydrocarbyl- -MeBmt- or -cis-MeBmt-residue wherein the hydrocarbyl moiety comprises at least two carbon atoms; or a -7'-desmethyl-7'-hydrocarbyl-dihydro-MeBmt- or -3'-O-acetyl-7'-desmethyl-7'-hydrocarbyl-dihydro-MeBmt- residue wherein the hydrocarbyl moiety comprises at least two carbon atoms and wherein any aliphatic group or moiety as or comprising said hydrocarbyl moiety is saturated; or
(iv) wherein the 3'-carbon atom of the residue at the 1-position is oxo, C_{1-4}alkoxyimino, azidoalkylcarbonyloxy or alkoxycarbonyloxy substituted, or wherein the β-carbon atom of the residue at the 2-position is β-oxo substituted or the residue at the 2-position is an (L)-isoleucyl residue; or
(v) of formula XI



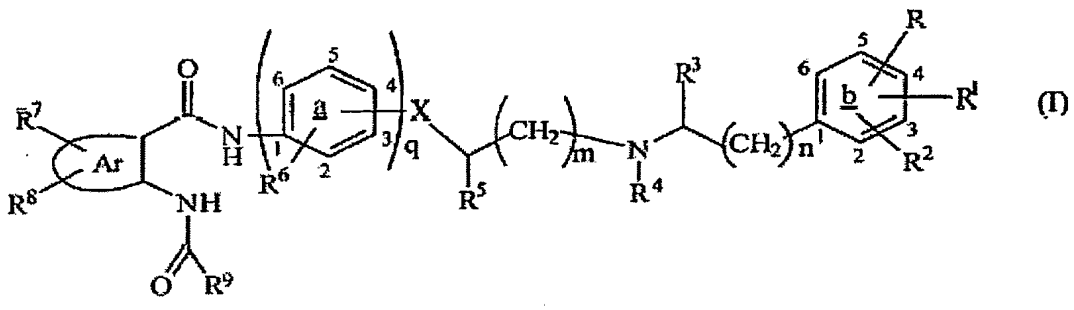
(XI)

wherein
A is -N-desmethyl-dihydro-MeBmt, B is -Thr- and Z is -MeVal-, or
A is -dihydro-MeBmt-, B is -Thr- and Z is -Val-, or
A is -MeLeu-, B is -αAbu- and Z is -Val-; or which is
(vi) a dicarboxylic acid di-ester of a cyclosporin having a β-hydroxy-(L)-α-amino acid residue at the 2-position.

Further derivatives as well as methods for producing said compound are detailed in EP-B1 0 296 122 which is, therefore, included herein in its entirety by reference.

Tariquidar (XR-9576); WO 98/17648

1. A compound which is an anthranilic acid derivative of formula (I):



(I)

wherein

each of R, R^1 and R^2, which are the same or different, is H, C_1-C_6 alkyl, OH, C_1-C_6 alkoxy, halogen, nitro, or $N(R^{10}R^{11})$ wherein each of R^{10} and R^{11}, which are the same or different, is H or C_1-C_6 alkyl, or R^1 and R^2, being attached to adjacent positions of ring b, together form a methylenedioxy or ethylenedioxy group;

R^3 is H or C_1-C_6 alkyl

R^4 is C_1-C_6 alkyl or R^4 represents $-CH_2-$ or $-CH_2CH_2-$ which is attached either (i) to position 2 of ring b to complete a saturated 5- or 6-membered nitrogen-containing ring fused to ring b, or (ii) to the position in ring a adjacent to that to which X, being a single bond, is linked, thereby completing a saturated 5- or 6-membered nitrogen-containing ring fused to ring a;

R^5 is H, OH or C_1-C_6 alkyl;

X is a direct bond, O, S, $-S-(CH_2)_p-$ or $-O-(CH_2)_p-$ wherein p is an integer of 1 to 6;

R_6 is H, C_1-C_6 alkyl or C_1-C_6 alkoxy;

q is 0 or 1;

Ar is an unsaturated carbocyclic or heterocyclic group;

each of R^7 and R^8, which are the same or different, is H, C_1-C_6 alkyl which is unsubstituted or substituted, C_1-C_6 alkoxy, hydroxy, halogen, phenyl, -NHOH, nitro, a group $N(R^{10}R^{11})$ as defined above or a group SR^{12} wherein R^{12} is H or C_1-C_6 alkyl; or R^7 and R^8, when situated on adjacent carbon atoms, form together with the carbon atoms to which they are attached a benzene ring or a methylenedioxy substituent;

R^9 is phenyl or an unsaturated heterocyclic group, each of which is unsubstituted or substituted by C_1-C_6 alkyl, OH, C_1-C_6 alkoxy, halogen, C_3-C_6 cycloalkyl, phenyl, benzyl, trifluoromethyl, nitro, acetyl, benzoyl or $N(R^{10}R^{11})$ as defined above, or two substituents on adjacent ring positions of the said phenyl or heterocyclic group together complete a saturated or unsaturated 6-membered ring or form a methylenedioxy group;

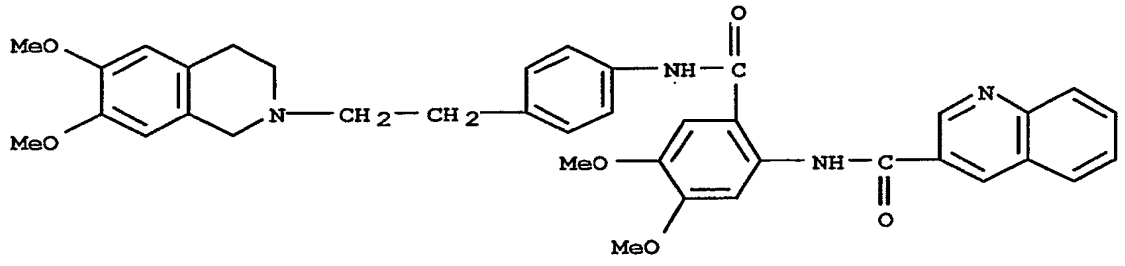
n is 0 or 1; and

m is 0 or an integer of 1 to 6;

or a pharmaceutically acceptable salt thereof.

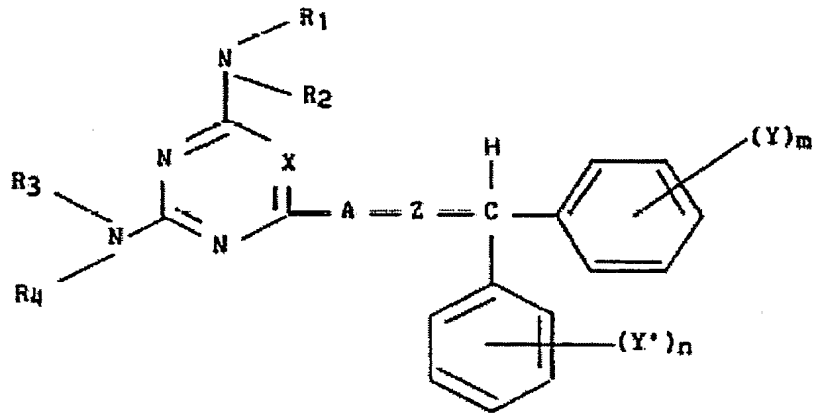
Further derivatives as well as methods for producing said compound are detailed in WO 98/17648 which is, therefore, included herein in its entirety by reference.

However,a compound of the following formula is preferred:



S-9788; EP-B1 0 466 586

Polymethyleneimines of the general formula I:

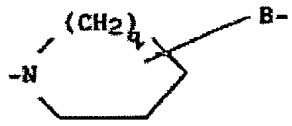


(I)

wherein:

a) X represents the group CH or a nitrogen atom;

b) A represents a polymethyleneimine group of the formula

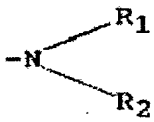


wherein:

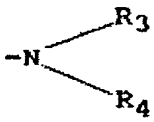
- B is a sulphur atom or a radical NR' wherein R' represents a hydrogen atom or a methyl radical,
  and
  g is 2;

c) each of R_1, R_2, R_3 and R_4, which are identical or different, represents:

- a hydrogen atom, an allyl radical or a propyl radical, with the proviso that at least one of the groups



and



represents an allylamino radical;

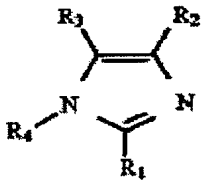
d) Z represents a methylene or ethylene radical;

e) each of Y and Y', which are identical or different, represents a hydrogen or fluorine atom or a methyl or methoxy radical;
f) each of m and n, which are identical or different, represents the integer 1 or 2;
and the corresponding diastereoisomers and enantiomers.

Further derivatives as well as methods for producing said compound are detailed in EP-B1 0 466 586 which is, therefore, included herein in its entirety by reference.

OC-144-093; US 5,756,527

Imidazol derivatives having formula 1



Formula I

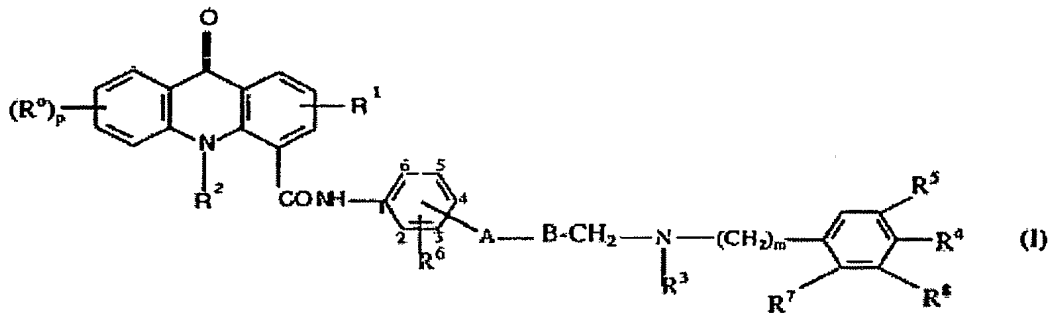
wherein:

R_1 is selected from the group consisting of: mono-,di-,and tri-substituted phenyl or thienyl, the substituents are selected from the group consisting of:
(i) substituted C_{1-6} alkyl, substituted C_{2-6} alkyloxy, wherein the substituents are selected from the group consisting of hydrogen or C_{1-6} alkoxy;
(ii) $C_{1-11}CO_2R_5$, trans-$CH{=}CHCO_2R_5$, wherein R_5 is C_{1-11} alkyl, or phenyl C_{1-11} alkyl;
R_2 and R_3 are mono-, di, and tri-substituted phenyl wherein the substituents are independently selected from:
(i) halo;
(ii) C_{1-6} alkyl-amino, or di(C_{1-6} alkyl)amino, and R_4 is hydrogen.

Further derivatives as well as methods for producing said compound are detailed in US 5,756,527 which is, therefore, included herein in its entirety by reference.

Elacridar (GF-120918); EP-A1 0 494 623

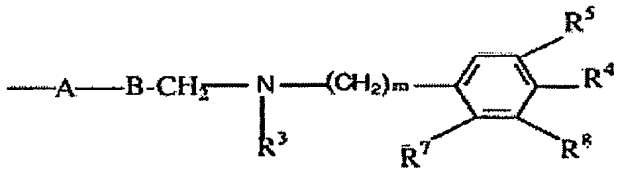
A compound of formula (I)



(I)

wherein R^0 represents a hydrogen or halogen atom, or a C_{1-4}alkyl, C_{1-4}alkoxy, C_{1-4}alkylthio, amino or nitro group;
p represents 1; or when R^0 represents C_{1-4}alkoxy may also represent 2 or 3;
R^1 represents a hydrogen or halogen atom, or a C_{1-4}alkyl, C_{1-4}alkoxy or C_{1-4}alkylthio group;
R^2 represents a hydrogen atom or a C_{1-4}alkyl group;
A represents an oxygen or a sulphur atom, a bond or a group $(CH_2)_1NR^9$ (where 1 represents zero or 1 and R^9 represents a hydrogen atom or a methyl group);
B represents a C_{1-4} alkylene chain optionally substituted by a hydroxyl group, except that the hydroxyl group and moiety A cannot be attached to the same carbon atom when A represents an oxygen or sulphur atom or a group $(CH_2)_1NR^9$, or when A represents a bond B may also represent a C_{2-4}alkenylene chain;
R^3 represents a hydrogen atom or a C_{1-4}alkyl group;
m represents 1 or 2;
R^4 represents a hydrogen or a halogen atom, or a C_{1-4}alkyl, C_{1-4}alkoxy or C_{1-4}alkylthio group;
R^5 represents a hydrogen atom or a C_{1-4}alkoxy group;
R^6 represents a hydrogen atom or a C_{1-4}alkyl or C_{1-4}alkoxy group;
R^7 represents a hydrogen atom or R^3 and R^7 together form a group $-(CH_2)_n-$where n represents 1 or 2;

R^8 represents a hydrogen atom or a C_{1-4}alkoxy group;
the group

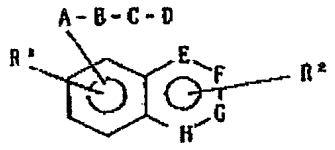


is attached at the benzene ring 3 or 4 position relate to the carboxamide substituent, provided that when the group is attached at the benzene ring 3 position then R^6 must be attached at the benzene ring 6 position;
and salts and solvates thereof.

Further derivatives as well as methods for producing said compound are detailed in EP-A1 0 494 623 which is, therefore, included herein in its entirety by reference.

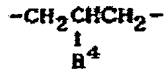
MS 209; US 5,405,843; EP 0 363 212 B1

A compound of the general formula [I]

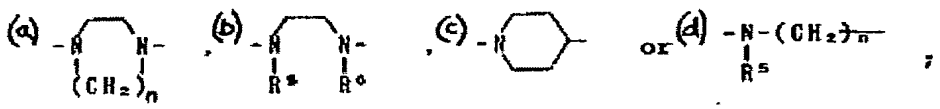


[I]

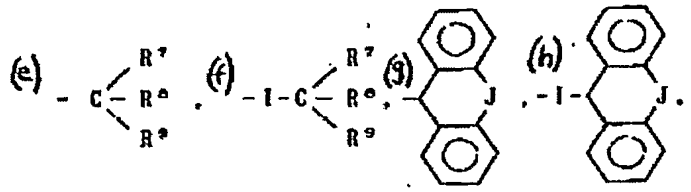
in which A represents an oxygen or sulfur atom or a methylene, amino or -NR³ group, which is bound to any available position on the condensed benzene ring; B represents -(CH₂)ₙ-,

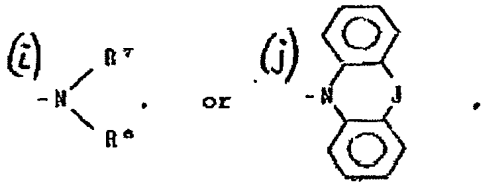


or -CO(CH₂)ₙ-; C represents

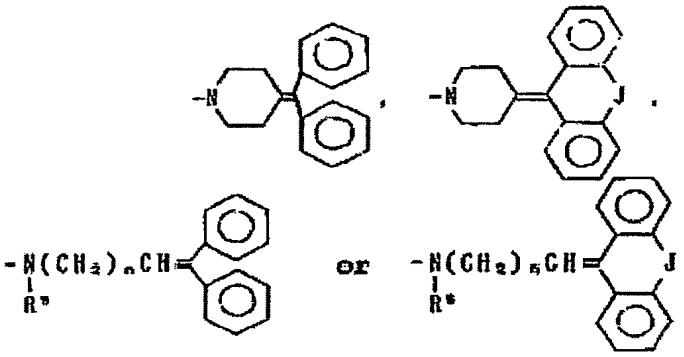


D represents





C and D can together form



E, F, G and H each independently represent a carbon or nitrogen atom, provided that either one or two of them is nitrogen, R^1 and R^2 each independently represent a hydrogen or halogen atom, a C_{1-4} alkyl, amino group, substituted amino group, a C_{1-4} alkoxy, C_{1-4} alkylthio, C_{1-4} alkylsulfonyl, trifluoromethyl, cyano, nitro, amide or hydroxy group, wherein R^1 and R^2 may be on any position available on the condensed ring or one each on each of the rings or both on the same ring of which the condensed ring is formed; R^3 represents a hydrogen atom or a C_{1-4} alkyl or acyl group; R^4 represents a hydroxyl, lower alkylamino (where alkyl is C_{1-4}), C_{1-4} alkoxyl or C_{1-2} acyloxy group; R^5 and R^6 each independently represent a hydrogen atom or a C_{1-4} alkyl or hydroxyalkyl group; R^7, R^8 and R^9 each independently represent a hydrogen atom or a hydroxy, phenyl, pyridyl or substituted phenyl group; I represents an oxygen atom,

or a nitrogen atom;

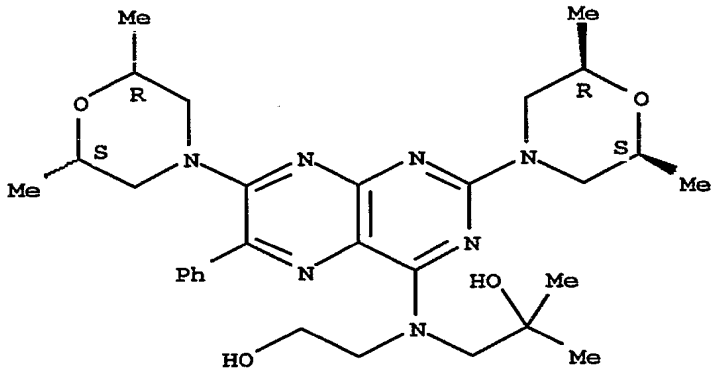
J represents $-(CH_2)_m-$, $-CH=CH-$, $-OCH_2-$ or an oxygen atom; n represents an integral number in the range between 1 and 10, and m represents an integral number, 0, 1 or 2, or a pharmaceutically acceptable salt thereof;

with the proviso that if C represents (a) or (b) then D does not represent (i) or (j) and I does not represent a nitrogen atom.

Further derivatives as well as methods for producing said compound are detailed in US 5,405,843 or EP 0 363 212 B1 which is, therefore, included herein in its entirety by reference.

BIBW-22; (DE 4225353)

A compound of the formula:



Further derivatives as well explanations of the indicated residues and methods for producing said compound are detailed in DE 4225353 which is, therefore, included herein in its entirety by reference.

Other inhibitors which are within the scope of the present invention are detailed e.g. in WO 98/48784 which is therefore incorporated herein by reference. It has to be understood that some of the inhibitor(s) mentioned before are identical with the following list of inhibitors which is exemplary and provides sufficient information concerning the chemical nature of the inhibitor(s) of the invention. In particular, WO 98/48784 discloses desmethoxyVerapamil, quinine, chinchonidine, primaquine, tamoxifen, dihydrocyclosporin, yohimbine, corynanthine, reserpine, physostigmine, acridine, acridine orange, quinacrine, trifluoroperazine chlorpromazine, propanolol, atropine, tryptamine, forskolin, 1 ,9-dideoxyforskolin, cyclosporin, (USPatent 4,117,118 (1978)), PSC-833 (cyclosporin D, 6-[(2S, 4R, 6E)-4-methyl-2 (methylamino)-3-oxo-6-octenoic acid]-(9Cl)), [US Patent 5,525,590] [ACS 121584-187], Keller et al., "SDZ PSC 833, a non-immunosuppressive cylcosporine: its

potency in overcoming p-glycoprotein-mediated multidrug resistance of murine leukemia", Int JCancer 50:593-597 (1992)), RU-486 (17 -hydroxy-11 p-[4-dimethylaminophenyl]-17a prop-l-ynyl estra-4, 9-dien-3 one), RU-49953 (17P-hydroxy-11P, 17a-[4dimethylaminophenyl] - 17a prop-l-ynyl estra-4, 9 dien-3 one), S9778 (6-{4-[2,2-di( ) ethylamino] - 1 -piperidinyl } -N, N ', di-2-propenyl- 1,3 ,5-triazine-2,4-diamine, bismethane sulfonate, [US patent 5,225,411; EP 466586] [ACS # 140945-01-3]; Dhainaut et al., "New triazine derivatives as potent modulators of multidrug resistance," J MedicinalChemistry 35:2481-2496 (1992)), MS-209 (5 -[3 -[4-(2,2-diphenylacetyl)piperazin- 1 -yl]2-hydroxypropoxy]quinoline sesquifumarate, [US patent 5,405,843 (continuation of 5,112,817)], [ACS # It 158681-49-3], Sato et al., "Reversal of multidrug resistance by a novel quinoline derivative, MS-209, Cancer Chemother Pharmacol 35:271-277 (1995)),MS-073 (Fukazawa et al., European Patent Application 0363212 (1989)), FK-506 (Tanaka et al., M. Physicochemical properties of FK-506, a novel immunosuppressant isolated from Streptomyces tsukubaensis" Transplantation Proceedings. 19(5 Suppl 6):1 1-6, (1987); Naito et al., "Reversal of multidrug resistance by an immunosuppressive agent FK-506," Cancer Chemother & Pharmacol. 29:195-200 (1992); Pourtier-Manzanedo et al., "FK-506 (fujimycin) reverses the multidrug resistance of tumor cells in vitro," Anti-Cancer Drugs 2:279-83 (1991); Epand & Epand, "The new potent immunosuppressant FK-506 reverses multidrug resistance inChinese hamster ovary cells," Anti-Cancer Drug Design 6:189-93 (1991)), VX-710 (2peperidinecarboxylic acid, 1-[oxo(3,4,5 -trimethoxyphenyl)acetyl] -3-(3-pyridinyl)-l- [3- (3-pyridinyl)propyl]butyl ester [ACS 159997-94-1] [US patent number 5,620,971]Germann et al., "Chemosensitization and drug accumulation effects of VX-710, Verapamil, cyclosporin A, MS-209 and GF120918 in multidrug resistance-associated protein MRP" Anti-Cancer Drugs 8, 141-155 (1997); Germann et al., "Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of Pglycoprotein-mediated multidrug resistance in vitro" Anti-Cancer Drugs 8, 125-140 (1997)), VX-853 ([US patent number 5,543,423] [ACS It 190454-58-1), AHC-52 (methyl 2-(N-benzyl-N-methylamino)ethyl-2, 6-dimethyl-4-(2-isopropylpyrazolo[1,5-a]pyridine-3-yl)-l ,4-dihyropyridine-3,5-dicarboxylate; [Japanese Patent 63 - 135381; European Patent 0270926] [ACS 119666-09-0] Shinoda et al., "In vivo circumvention of vincristine resistance in mice with P388 leukemia using a novel

compound, AHC-52,"Cancer Res 49:1722-6 (1989)), GF-120918 (9,10-dihydro-5-methoxy-9-oxo-N-[4-[2- (1,2,3 ,4-tetrahydro-6,7-dimethoxyisoquinol-2-yl) ethyl]phenyl]-4 acridinecarboxamide,[US patent 5,604,237] [ACS It 143664-11-3] Hyafil et al., "In vitro and in vivo reversal of multidrug resistance by GF 120918, an acridonecarboxamide derivative," Cancer Res 53:4595-4602 (1993)), and XR-9051 (3-[(3Z, 6Z)-6Benzylidene- 1 -methyl-2 ,5 -dioxopiperazin-3 -ylideneмethyl] -N-[4-[2-(6,7-dimethoxy- 1,2,3 ,4-tetrahydroisoquinolin-2-yl)ethyl]phenyl]benzamide hydrochloride, [AC SIt57-22- 7]). Other inhibitors are for example listed in WO 99/17757, which is therefore incorporated herein by reference. As mentioned above, reference may be made to the literature for sources of appropriate ABC-transporter inhibitors to use according to the invention.

Thus, it has to be understood that also other ABC-transporter inhibitors which fulfill the above criteria (i.e. which are capable of reducing the transport of hyaluronan across a lipid bilayer as mediated by ABC-transporter(s)) are within the scope of the present invention, as are their derivatives e.g. functional derivatives and analogues, and any isomers (e.g. stereoisomers and/or enantiomers) thereof. Also included are the salts of the inhibitor(s) as mentioned herein, including both organic and inorganic salts (e.g. with alkali and alkaline earth metals, ammonium, ethanolamine, diethanolamine and meglumine, chloride, hydrogen carbonate, phosphate, sulphate and acetate counterions). Appropriate pharmaceutically acceptable salts are well described in the pharmaceutical literature. In addition, some of these salts may form solvates with water or organic solvents such as ethanol. Such solvates are also included within the scope of this invention.

In another aspect of the methods and uses of the present invention, the inhibitor is an antibody, preferably an antibody the binding of which interferes with the transport of hyaluronan mediated by the ABC-transporters of this invention. It is envisaged that the antibody specifically recognizes the ABC-transporter to whom it is directed to, i.e. the antibody shows no or essentially no cross-reactivity to other proteins and/or other ABC-transporter(s). For example a monoclonal antibody against P-glycoprotein (C219 from Calbiochem) [70] was used to verify the participation of the MDR transporter in hyaluronan export. Membranes from this cell line were

incubated with and without the antibody and then assayed for hyaluronan transport activity. The antibody decreased the hyaluronan transport activity by 20%. Other antibodies which are within the gist of the present invention are well known to the skilled person and are exemplified by the following non-limiting selection: Anti-P-Glycoprotein (4E3), Human (Mouse); Anti-P-Glycoprotein (C219), Hamster and Human (Mouse); Anti-P-Glycoprotein (C494), Hamster and Human (Mouse); Anti-P-Glycoprotein (JSB-1), Hamster (Mouse); Anti-P-Glycoprotein (4E3), Human (Mouse); Anti-P-Glycoprotein (C219), Hamster and Human (Mouse); Anti-P-Glycoprotein (C494), Hamster and Human (Mouse); Anti-P-Glycoprotein (JSB-1), Hamster (Mouse); Anti-P-Glycoprotein, Human (Rabbit). All the above listed antibodies are manufactured by Calbiochem. Other antibodies which are specific for an ABC-transporter can be easily produced by methods well-known in the art. For example it is possible to use cell lines secreting antibody to essentially any desired substance that produces an immune response. RNA encoding the light and heavy chains of the immunoglobulin can then be obtained from the cytoplasm of the hybridoma. The 5' end portion of the mRNA can be used to prepare cDNA to be inserted into an expression vector. The DNA encoding the antibody or its immunoglobulin chains can subsequently be expressed in cells, preferably mammalian cells. Depending on the host cell, renaturation techniques may be required to attain proper conformation of the antibody. If necessary, point substitutions seeking to optimize binding or stability of the antibody may be made in the DNA using conventional cassette mutagenesis or other protein engineering methodology such as is disclosed herein. Furthermore, antibodies or fragments thereof to the aforementioned ABC-transporter(s) can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. Other suitable antibodies as well as methods for testing the effectivness of such antibodies are detailed in WO02071061.

For the production of antibodies in experimental animals, various hosts including goats, rabbits, rats, mice, and others, may be immunized by injection with polypeptides of the present invention or any fragment or oligopeptide or derivative thereof which has immunogenic properties. Techniques for producing and processing polyclonal antibodies are known in the art and are described in, among

others, Mayer and Walker, eds., "Immunochemical Methods in Cell and Molecular Biology", Academic Press, London (1987). Polyclonal antibodies also may be obtained from an animal, preferably a mammal, previously infected with the virus of the invention. Methods for purifying antibodies are known in the art and comprise, for example, immunoaffinity chromatography. Depending on the host species, various adjuvants or immunological carriers may be used to increase immunological responses. Such adjuvants include, but are not limited to, Freund's, complete or incomplete adjuvants, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions and dinitrophenol. An example of a carrier, to which, for instance, a peptide of the invention may be coupled, is keyhole limpet hemocyanin (KLH). When derivatives of said antibodies are obtained by the phage display technique, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies which bind to an epitope of the peptide or polypeptide of the invention (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13). In many cases, the binding phenomena of antibodies to antigens is equivalent to other ligand/anti-ligand binding.

The production of chimeric antibodies is described, for example, in WO89/09622. A further source of antibodies to be utilized in accordance with the present invention are so-called xenogenic antibodies. The general principle for the production of xenogenic antibodies such as human antibodies in mice is described in, e.g., WO 91/10741, WO 94/02602, WO 96/34096 and WO 96/33735.

The production of recombinant antibodies is described, for example, in R. Kontermnn, S. Dübel: Antbody Engineering, Springer Lab Manual 2001.

In a preferred embodiment, the antibody of the invention has an affinity of at least about 10^{-7} M, preferably at least about 10^{-8} M more preferably at least about 10^{-9} M and most preferably at least about 10^{-10} M.

The antibody which is used in accordance with the uses or methods of the invention may be a monoclonal or a polyclonal antibody (see Harlow and Lane, "Antibodies, A

Laboratory Manual", CSH Press, Cold Spring Harbor, USA, 1988) or a derivative of said antibody which retains or essentially retains its binding specificity. Preferred derivatives of such antibodies are chimeric antibodies comprising, for example, a mouse or rat variable region and a human constant region.

The term "specifically recognizing" in connection with the antibody used in accordance with the present invention means that the antibody etc. does not or essentially does not cross-react with polypeptides of similar structures. Cross-reactivity of a panel of antibodies etc. under investigation may be tested, for example, by assessing binding of said panel of antibodies etc. under conventional conditions to the polypeptide of interest as well as to a number of more or less (structurally and/or functionally) closely related polypeptides. Only those antibodies that bind to the polypeptide of interest (i.e to an ABC-transporter which transports hyaluronan across a lipid bilayer) but do not or do not essentially bind to any of the other polypeptides which are preferably expressed by the same tissue as the polypeptide of interest (e.g. in a chondrocyte or in cells which are comprised in cartilage and which are responsible for the hyaluronan synthesis) are considered specific and are, therefore, selected for further studies in accordance with the invention.

The term "functional fragment" as used herein refers to fragments of the antibodies as specified herein which retain or essentially retain the binding specificity of the antibodies like, separated light and heavy chains, Fab, Fab/c, Fv, Fab', F(ab')2. The term "antibody" also comprises bifunctional antibodies and antibody constructs, like single chain Fvs (scFv) or antibody-fusion proteins. The term "scFv fragment" (single-chain Fv fragment) is well understood in the art and preferred due to its small size and the possibility to recombinantly produce such fragments. It is also envisaged in context of this invention that the term "antibody" comprises antibody constructs which may be expressed in cells, e.g. antibody constructs which may be transfected and/or transduced via, inter alia, viruses or vectors. It is in particular envisaged that such antibody constructs specifically recognize the polypeptides of the present invention. It is, furthermore, envisaged that said antibody construct is employed in gene therapy approaches.

In a particularly preferred embodiment of the method of the invention, said antibody

or antibody binding portion is or is derived from a human antibody or a humanized antibody. The term "humanized antibody" means, in accordance with the present invention, an antibody of non-human origin, where at least one complementarity determining region (CDR) in the variable regions such as the CDR3 and preferably all 6 CDRs have been replaced by CDRs of an antibody of human origin having a desired specificity. Optionally, the non-human constant region(s) of the antibody has/have been replaced by (a) constant region(s) of a human antibody. Methods for the production of humanized antibodies are described in, e.g., EP-A1 0 239 400 and WO90/07861.The specifically binding antibody etc. may be detected by using, for example, a labeled secondary antibody specifically recognizing the constant region of the first antibody. However, in a further particularly preferred embodiment of the method of the invention, the antibody or derivative of said antibody itself is detectably labeled at the binding portion. Detectable labels include a variety of established labels such as radioactive (^{125}I, for example) or fluorescent labels (see, e.g. Harlow and Lane, loc. cit.). Binding may be detected after removing unspecific labels by appropriate washing conditions (see, e.g. Harlow and Lane, loc. cit.).

In addition to ABC-transporter antibodies and functional antibody fragments, small molecule peptidomimetics or non-peptide mimetics can be designed to mimic the action of the ABC-transporter antibodies in inhibiting or modulating the transport of hyaluronan, presumably by interfering with the action of said ABC-transporter(s). Methods for designing such small molecule mimics are well known (see, for example, Ripka and Rich, Curr. Opin. Chem. Biol. 2: 441-452, 1998; Huang, et al., Biopolymers 43: 367-382,1997; al-Obeidi, et al., Mol. Biotechnol. 9: 205-223, 1998). Small molecule inhibitors that are designed based on the ABC-transporter antibody may be screened for the ability to interfere with the ABC-transporter – ABC-transporter-antibody binding interaction. Candidate small molecules exhibiting activity in such an assay may be optimized by methods that are well known in the art, including for example, *in vitro* screening assays, and further refined in *in vivo* assays for inhibition or modulation of ABC-transporter-mediated hyaluronan-transport by any of the methods described herein or as are well known in the art. Such small molecule inhibitors of the ABC-transporter action (transport of hyaluronan) should be useful in the present uses and/or methods for treating and/or

preventing arthritis and/or for screening compounds which might be useful as new lead compounds for the generation of more effective ABC-transporter inhibitors.

In another preferred embodiment of the uses and methods of the present invention, the inhibitor is an anti-sense, iRNA, siRNA or ribozyme. An siRNA approach is, for example, dislosed in Elbashir ((2001), Nature 411, 494-498)). It is also envisaged in accordance with this invention that for example short hairpin RNAs (shRNAs) are employed in accordance with this invention as inhibitors. The shRNA approach for gene silencing is well known in the art and may comprise the use of st (small temporal) RNAs; see, inter alia, Paddison (2002) Genes Dev. 16, 948-958. Approaches for gene silencing are known in the art and comprise "RNA"-approaches like RNAi or siRNA. Successful use of such approaches has been shown in Paddison (2002) loc. cit., Elbashir (2002) Methods 26, 199-213; Novina (2002) Mat. Med. June 3, 2002; Donze (2002) Nucl. Acids Res. 30, e46; Paul (2002) Nat. Biotech 20, 505-508; Lee (2002) Nat. Biotech. 20, 500-505; Miyagashi (2002) Nat. Biotech. 20, 497-500; Yu (2002) PNAS 99, 6047-6052 or Brummelkamp (2002), Science 296, 550-553. These approaches may be vector-based, e.g. the pSUPER vector, or RNA polIII vectors may be employed as illustrated, inter alia, in Yu (2002) loc. cit.; Miyagishi (2002) loc. cit. or Brummelkamp (2002) loc. cit. "Anti-sense" and "antisense nucleotides" means DNA or RNA constructs which block the expression of the naturally occurring gene product. As used herein, the terms "antisense oligonucleotide" and "antisense oligomer "are used interchangeably and refer to a sequence of nucleotide bases that allows the antisense oligomer to hybridize to a target sequence in an RNA by Watson Crick base pairing, to form an RNA: oligomer heteroduplex within the target sequence. The term "target sequence" in this regard refers to the sequence of the ABC-transporters as described herein; the Accession numbers of the human ABC-transporters are e.g. depicted in **Figure 1**; other (e.g. non-human) ABC-transporters are well known to the skilled person and can be easily searched for example on http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed. The oligomer may have exact sequence complementarity to the target sequence or near complementarity. Such antisense oligomers may block or inhibit translation of the mRNA containing the target sequence, or inhibit gene transcription, may bind to double-stranded or single

stranded sequences. Preferably, said antisense oligonucleotides as used herein are "nuclease-resistant" oligomeric molecule e.g. their backbone is not susceptible to nuclease cleavage of a phosphodiester bond. Exemplary nuclease resistant antisense oligomers are oligonucleotide analogs, such as phosphorothioate and phosphate-amine DNA (pnDNA), both of which have a charged backbone, and methylphosphonate, morpholino, and peptide nucleic acid (PNA) oligonucleotides, all of which may have uncharged backbones.

In accordance with the present invention, the term "aptamer" means nucleic acid molecules that can bind to target molecules. Aptamers commonly comprise RNA, single stranded DNA, modified RNA or modified DNA molecules. The preparation of aptamers is well known in the art and may involve, inter alia, the use of combinatorial RNA libraries to identify binding sides (Gold, Ann. Rev. Biochem. 64 (1995), 763-797).

Arthritis is, as mentioned before, accompanied with a loss of cartilage at the joint surface. The cartilage goes through different stages during pathogenesis. At first chondrocytes try to replace loss of cartilage by increased synthesis and proliferation; simultaneously lacunae of edema and increased water binding occurs which leads to softening of the cartilage matrix. At the second stage new cartilage production cannot compensate for the loss and at the third stage loss of cartilage is complete.

Thus, in a further embodiment of the uses and methods of the present invention said arthritis is characterized by a degeneration and/or a destruction of cartilage. The term "degeneration and/or destruction of cartilage" includes within the meaning of the present invention dysregulation of turnover and repair of joint tissue. The pathological features are focal areas of destruction of articular cartilage associated with hypertrophy of the subcondral bone, joint margin and capsule. The radiological changes include joint space narrowing, subchondral sclerosis and cysts, pain, loss of joint motion and disability.

In a further embodiment of the methods and uses of the present invention said arthritis is osteoarthritis, (juvenile) chronic arthritis, rheumatoid arthritis, psoriatic arthritis, *A. mutilans*, septic arthritis, infectious arthritis and/or reactive arthritis. Thus, the term "arthritis" as used herein includes all forms of arthritis. First, the primary or idiopathic form. The secondary forms such as metabolic disorders such as ochronosis, acronmegaly, hemochromatosis and calcium crystal deposition and gout or apatite deposition; anatomic derangements such as slipped epiphysis, epiphysial dysplasias, Blount´s disease, Legge-Perthe disease, congetial dislacation of the hip, leg lenght inequality, hypermobility syndromes; traumatic causes such as major joint trauma, fracture through a joint or osteonecrosis, joint surgery, chronic injury; any inflammatory arthropathy such as (juvenile) chronic arthritis, rheumatoid arthritis, psoriatic arthritis, A. mutilans, septic arthritis, infectious arthritis and/or reactive arthritis; joint abnormalities in thyroid diseases, diabetes melitus, hemophilia, amyloidosis, dialysis arthropathies, primary hyperlipidemias and xanthpmatosis, Gaucher´s disease, mucopolysaccharidosis; metabolic, regional and heritable bone and joint diseases such as osteoporosis, osteomalacia, renal bone diseases, algodystrophy/reflex sympathetic dystrophy syndrom, Paget´s disease, hypertrophic osteoarthopathy, tumors of bone, heritable collagen disorders, hypermobility syndrome, joint dysplasias [96]. The respective diseases as well as their symptoms are well-known to the skilled person and e.g. derivable from textbooks like Pschyrembel et al. or the like.

The inhibitors of the present invention can be applied prophylactically with subjects that have or might have an enhanced individual risk factors such as obesity, heredity, for women after the menopause, osteoporosis, hypermobility, for persons with distorted joint shape or for persons with repetitive use of particular joint groups.

Thus in a further embodiment of the uses of the present invention said inhibitor(s) is(are) to be administered prophylactically.

Alternatively, the inhibitors can by applied therapeutically as early as possible e.g. after diagnosis of joint insult to inhibit further destruction, to support self regeneration and restore joint function.

Thus, in another embodiment of the uses of the present invention said inhibitor(s) is(are) to be administered therapeutically.

The dosage regimen utilising the inhibitors or screened compounds(inhibitors) of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; and the particular compound employed. It will be acknowledged that an ordinarily skilled physician or veterinarian can easily determine and prescribe the effective amount of the compound required to prevent, counter or arrest the progress of the condition.

It is also envisaged that the inhibitors of the present invention are employed in co-therapy approaches, i.e. in co-administration with other medicaments or drugs, for example other drugs for preventing, treating or ameliorating arthritis which are known in the art (e.g. hyaluronan injections like Hyalgan ® (Sanofi Pharmaceuticals), Orthovisc ® (Anika Therapeutics) and SynVisc ® (Biomatrix, now Genzyme), anti-inflammatory drugs and so on). It will be appreciated that these list of co-administered drugs is not limiting but severs as an example only. The skilled person is of course well-aware of suitable drugs which have a beneficial effect on arthritis and, therefore, might be useful when co-administered with the inhibitor(s) as described herein.

The combination of other drugs with the ABC transport inhibitors will be particularly important for the secondary forms of arthritis described above, since in theses cases they can optimally exert their beneficial properties, if the primary cause of cartilage destruction is also eliminated. For septic arthritis it should be combined with antibiotics, for the inflammatory forms of arthritis with corticosteroids and immunosuppressives.

Based on the finding that the inhibition of an ABC-transporter specifically reduces or abolishes the transport of hyaluronan across a lipid bilayer as mentioned before, the methods of the invention allow the convenient identification and/or isolation of

compounds that counteract the transport of hyaluronan mediated by ABC-transporters such that a normal transport of hyaluronan is restored or essentially restored. The meaning of a "normal" transport of hyaluronan was explained herein above. Thus, the present invention opens up the possibility to screen for compounds which reduce the transport of hyaluronan as mediated by ABC-transporter(s) in a cell/tissue/subject and, thereby, are suitable for treating or preventing arthritis in a subject. These methods can be applied to screen for efficient drugs for the treatment/prevention of arthritis.

The term a compound "which is suitable for the treatment of arthritis" as used herein defines a compound which prevents or ameliorates arthritis, i.e. which has a beneficial effect (e.g. reduction of the overall transport of hyaluronan across a lipid-bilayer; reduction of the destruction of cartilage; maintenance of the actual state of destruction of cartilage; prevention of a further destruction of the cartilage etc.). The term "arthritis" has been defined elsewhere in this specification.

Suitable test-assays for measuring the specific inhibition of the hyaluronan transport as mediated by ABC-transporter(s) can be carried out as follows. However, it has to be understood that these methods are exemplary only and are not intended to limit the scope of the present invention.

The specific transport can be measured in single cells by introducing labelled hyaluronan into the cell, e.g. by microinjection or otherwise as described herein and, e.g in [115]. A suitable label is e.g. a fluorescent tag. However, also other tags (described elsewhere herein) may be used. It is also envisaged to use derivatives and the like of hyaluronan. Said derivatives which can be used instead of hyaluronan have been described elswhere in this specification. After injection into the cell, e.g the disappearance of fluorescence under the influence of hyaluronan transport inhibitors from the cytosol can directly be observed by a fluorescence microscope. The quantification of other labels will depend on the respective label used. The corresponding methods for detecting such labels are well-known in the art. As mentioned elsewhere before, it is also envisaged to employ cells which *per se* do not express/contain hyaluronan synthase, but which contain one or more

ABC-transporter(s). It is, however, also envisaged to use cells which contain a hyaluronan-synthase – in such particular cases, the hyaluronan synthase can be inhibited e.g. by suitable antisense constructs, iRNA, siRNA, antibodies and/or other inhibitors which are well known to the skilled artisan.

Another specific screening method for inhibitors of hyaluronan transport is based on the introduction of partially degraded [^{14}C]hyaluronan into a cell line, preferably a human cell line by lipofectamine. It will be appreciated that of course other lipid formulations such as lipofectin, the SuperFect Transfection Reagent from Qiagen, DOPSA or DOPE are similarly applicable.

[^{14}C]hyaluronan is obtained by enzymatic synthesis from streptococcal membranes. The group C Streptococcus D181 strain is grown over night in TH-medium, diluted with fresh medium at a ratio of 1:3 and grown for another 3 hours. A suspension of the bacteria (10 ml) are subjected to ultrasonification (1 min 40 Watts) to disrupt the cells and sedimented by centrifugation at 10.000 g for 5 min. The sediment is washed with 50 mM TRIS-malonate pH 7.0 and incubated with 0.3 ml of substrate for hyaluronan synthesis (160 µM UDP-GlcNac and 8 µM UDP-[^{14}C]GlcA (specific activity 320 mCi/mmol, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.15 M NaCl) and incubated for 4 h at 37°C. [^{14}C]hyaluronan labelled hyaluronan is separated by gel filtration on a short column of Sephadex G-25 with the serum reduced medium Opti-MEM I from GIBCO as eluant and has a total radioactivity of 360.000 cpm. This solution was subjected to ultrasonification (1 min 40 Watts) to fragment the labelled hyaluronan.

Human cells are plated into microtiter plates and grown to 80-100% confluence. The medium was then changed to the solution of [^{14}C]hyaluronan in Opti-MEM I as prepared above containing 5-10 µg/ml of lipofectamine and incubated for various times (1 - 72 hours) (100 µl/well). The cells are rinsed thoroughly and incubated in DMEM, 10% foetal calf serum. After different time periods 50 µl of the medium is withdrawn and its radioactivity is determined.

The screening methods of the present invention can, in general, be categorized into different groups, which, however does not mean that the methods of the present invention are also limited to the following particular groups. These and other

methods are explained and exemplified in the following sections as well as in the appended examples.

1. Determination of hyaluronan transport activity in isolated membranes of cells.
2. Assay of the hyaluronan concentration in media of cell cultures or organ cultures e.g. by radioactive incorporation of [3H]glucosamine, by ELISA or chemical assays of extracted hyaluronan.
3. Histological or immunohistological staining of hyaluronan in organ cultures or in tissues obtained from animal trails.

In order to demonstrate the specificity of the hyaluronan transport as mediated by ABC-transporter(s), i.e. to demonstrate that the mentioned ABC-transporter(s) is(are) mainly responsible for the absence/reduction of hyaluronan in the exterior of a cell (e.g. comprised in cell culture or in organ culture), it is envisaged that the respective cell culture or organ culture is pretreated with an inhibitor(s) of the hyaluronan synthase. Accordingly, any further reduction of the hyaluronan (or the respective analogues/derivatives of hyaluronan as described elsewhere) is specifically due to the reduction of the hyaluronan transport activity as mediated by one or more ABC-transporter(s). Alternatively, it is possible to determine the hyaluronan transport of a cell which is treated with an inhibitor of the hyaluronan synthase with the hyaluronan transport of the same type of cell (e.g. a chondrocyte) which is treated with such inhibitor(s) of hyaluronan synthase together with an inhibitor of the present invention. The comparison of the hyaluronan transport rate of both cells will allow the determination of the effect of the respective inhibitor of the ABC-transporter which was tested. Inhibitor(s) of hyaluronan synthase are well-known to the skilled artisan, e.g form [24-29]. Examles of such inhibitor(s) are exemplified in the following: Periodate-oxidized UDP-Glucuronic acid, periodate-oxidized UDP-N-acetyl-glucosamine [24,25]. Alternatively, it is also possible to reduce the content of hyaluronan synthase within the test-cell, e.g. by antisense, iRNA, siRNA and so on as described in [10]. The respective sequence of the hyaluronan synthase are well known to the skilled artisan. There are three human hyaluronan synthase with the Accession numbers NP_005319 for hyaluronan

synthase 2, NP_619515 for hyaluronan synthase 3 isoform b, NP_005320 for hyaluronan synthase 3 isoform a, NP_001514 for yaluronan synthase 1.

In one embodiment, the present invention relates to a method for screening a compound which is suitable for the treatment of arthritis, said method comprising:

(a) contacting an isolated lipid bilayer comprising at least one ABC-transporter which is capable of transporting hyaluronan with a test compound and an indicator compound;

(b) measuring the effect of the test compound on the transport of the indicator compound across the lipid bilayer; and

(c) identifying test compounds which reduce the transport of the indicator compound.

The term "compound" which is interchangeable with "test compound" in accordance with the screening methods of the present invention shall mean any biologically active substance that has an effect on the transport of hyaluronan as mediated by ABC-transporter(s), whereas such compound has a positive or negative influence upon such ABC-transporter mediated hyaluronan-transport across a lipid bilayer. Preferred compounds are nucleic acids, preferably coding for a peptide, polypeptide, antisense RNA, iRNA, siRNA or a ribozyme or nucleic acids that act independently of their transcription respective their translation as for example an antisense RNA or ribozyme; natural or synthetic peptides, preferably with a relative molecular mass of about 1.000, especially of about 500, peptide analogs polypeptides or compositions of polypeptides, proteins, protein complexes, fusion proteins, preferably antibodies, especially murine, human or humanized antibodies, single chain antibodies, F_{ab} fragments or any other antigen binding portion or derivative of an antibody, including modifications of such molecules as for example glycosylation, acetylation, phosphorylation, farnesylation, hydroxylation, methylation or esterification, hormones, organic or inorganic molecules or compositions, preferably small molecules with a relative molecular mass of about 1.000, especially of about 500.

The screening for inhibitors that are specific for a particular ABC transporter and that do not inhibit the synthase can be performed as already described above for HT29 and HT29-mdr. The gene for the ABC-transporter is cloned and transfected into a recipient cell line that produces hyaluronan. Both cell lines are then compared for their responsiveness toward the compound to be tested. Since the cell lines differ only in the overexpression of a certain ABC transporter, any difference in the response of the hyaluronan transport activity or in hyaluronan in increasing concentrations of the test compound can be attributed to a specific interaction between the inhibitor and the expressed transporter. Methods for over-expressing ABC-transporter(s) are well-known in the art and, additionally exemplified in this specification. Provided that the used test-cell comprises further ABC-transporter(s) it is envisaged that these ABC-transporter(s) is(are) identified (e.g. by antibody-mediated FACS-analysis or by RT-PCR as explained herein) and subsequently disrupted/blocked by e.g. antisense-constructs, iRNA, siRNA, antibodies and so on in order to specifically address the reduction of the hyaluronan transport to the over-expressed ABC-transporter. It is also envisaged that the hyaluronan synthase which might be active in these cells is blocked/disrupted e.g. by any of the above mentioned methods (antisense, iRNA and so on).

An alternative method is the following procedure: If a transporter has been identified as a hyaluronan transporter that in addition to hyaluronan also transports other substrates (a hypothesis that is very likely regarding the known broad substrate specificities of the transporters), it is very easy to find substrate analogs that will be transported. These substrate analogs are preferably labelled and can serve as indicators or probes for the inhibitory action of compounds to be tested. In fact, this scenario was already the starting point for the identification of inhibitors for hyaluronan production described here. Suitable labels and hyaluronan analogs have been described elsewhere in this specification.

It is furthermore envisaged that the screening-methods described herein (e.g. the screening methods for screening a compound/inhibitor which is suitable for the treatment of arthritis) are carried out in the form of high-throughput methods. Such methods for screening for transdominant effector peptides and RNA molecules are

for example described in WO 97/27213 or WO 97/27212 or EP-B1 0 832 207. Thus it has to be understood that the present invention also encompasses methods for screening for an inhibitor which is (i) capable of altering the hyaluronan-transporting phenotype of a cell (i.e. the inhibitor effects the hyaluronan-transport mediated by one or more ABC-transporter(s)) and thereby represents an inhibitor which is (ii) suitable for the treatment and/or prevention of arthritis. The methods comprise the steps of a) introducing a molecular library of randomized candidate nucleic acids into a plurality of test-cells, wherein each of said nucleic acids comprises a different nucleotide sequence; b) screening the plurality of test-cells for a cell exhibiting an altered phenotype, wherein the altered phenotype is due to the presence of an inhibitor. The methods may also include the steps of c) isolating the test-cell(s) exhibiting an altered phenotype; and d) isolating a candidate nucleic acid from the cell(s). The introduction (e.g. by way of retroviral vectors) and construction of suitable molecular libraries of randomized candidate nucleic acids is detailed, e.g. in WO 97/27213. The test-cells to be used in such systems are preferably devoid of hyaluronan-synthase or, alternatively, the hyaluronan-synthase is inhibited as exemplified above (antisense; iRNA, and so on). Furthermore, it is envisaged that the test-cells over-express one or more ABC-transporter(s) in order to mimic an arthritic phenotype. The hyaluronan-transporting phenotype may be measured as described herein e.g. introducing labelled hyaluronan or hyaluronan-derivatives, -analogues and the like into the test-cell and measuring the amount of hyaluronan (e.g. fluorescent labelled hyaluronan) before and after the introduction (and/or expression) of said library of randomized candidate nucleic acids. It is also envisaged that the library of randomized candidate nucleic acids is under the control of a regulatable promoter (described elsewhere herein) which hallows a precise control of the experimental conditions.

Compounds tested positive for being capable of reducing the hyaluronan transport across a lipid bilayer are prime candidates for the direct use as a medicament or as lead compounds for the development of a medicament. Naturally, the toxicity of the compound identified and other well-known factors crucial for the applicability of the compound as a medicament will have to be tested. Methods for developing a

suitable active ingredient of a pharmaceutical composition on the basis of the compound identified as a lead compound are described elsewhere in this specification.

The term "indicator compound" within the meaning of the present invention relates to hyaluronan that has been metabolically labelled in Streptococci or eukaryotic cells with [^{3}H]glucosamine or [^{14}C]glucose [41,42,69]; hyaluronan that has been labelled during synthesis from membranes of Streptococci or eukaryotic cells in vitro by incubation with UDP-N-acetyl-[^{3}H]glucosamine or UDP-[^{14}C]glucuronic acid [41,42,69]; Oligosaccharides of hyaluronan that have been radioactively labelled by reduction with $NaB^{3}H_4$ [103]; hyaluronan or oligosaccharides of hyaluronan that have been biotinylated [99]; hyaluronan or oligosaccharides of hyaluronan that have been made fluorescent with rhodamine or fluorescamine [100]; hyaluronan or oligosaccharides of hyaluronan that have been iodinated [101].

Suitable labels are known in the art and include radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99}mTc), and fluorescent labels, such as fluorescein and rhodamine, and biotin. Metabolic labelling of hyaluronan or labelling in direct enzyme assay is preferable performed with carbon (^{14}C) and tritium (^{3}H) [97]. Hyaluronan can also be quantified by colour reactions such as the carbazol reaction [98] or by sensitive assays utilizing hyaluronan labelled with biotin [99], fluorescent groups [100], iodine[101].

In another embodiment, the present invention relates to a method for screening a compound which reduces the transport of hyaluronan mediated by (an) ABC-transporter(s), said method comprising:

(a) contacting an isolated lipid bilayer comprising at least one ABC-transporter which is capable of transporting hyaluronan with a test compound and an indicator compound;

(b) measuring the effect of the test compound on the transport of the indicator compound across the lipid bilayer; and

(c) identifying test compounds which reduce the transport of the indicator compound.

In a further embodiment, the present invention relates to a method of screening for a compound which is suitable for the treatment of arthritis, said method comprising:

(a) contacting a cell comprising at least one ABC-transporter which is capable of transporting hyaluronan with a test compound and an indicator compound;

(b) measuring the effect of the test compound on the transport of the indicator compound across a lipid bilayer of the cell; and

(c) identifying compounds which reduce the transport of the indicator compound.

In a further embodiment, the present invention relates to a method for screening a compound which reduces the transport of hyaluronan mediated by (an) ABC-transporter(s), said method comprising:

(a) contacting a cell comprising at least one ABC-transporter which is capable of transporting hyaluronan with a test compound and an indicator compound;

(b) measuring the effect of the test compound on the transport of the indicator compound across a lipid bilayer of the cell; and

(c) identifying compounds which reduce the transport of the indicator compound.

In a preferred embodiment of the inventive methods, said screened compound specifically reduces the transport of hyaluronan mediated by said ABC-transporter. The meaning of the term "specifically reduces" was already explained herein above.

It is envisaged that the cell can be a bacterial, an insect, a fungal, or an animal cell.

The term "bacterial" is meant to include all bacteria which can be transformed or transfected with a DNA or RNA molecules for the expression of one or more ABC-transporter(s). Bacterial hosts may include gram negative as well as gram positive bacteria such as, for example, E. coli, S. typhimurium, Serratia marcescens and Bacillus subtilis. A polynucleotide sequence encoding one or more ABC-transporter(s) can be used to transform or transfect the cell using any of the techniques commonly known to those of ordinary skill in the art. Furthermore, methods for preparing fused, operably linked genes and expressing them in, e.g., mammalian cells and bacteria are well-known in the art (Sambrook, Molecular

Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

In one embodiment of the methods of the invention, said animal cell is a mammalian cell or a mammalian cell line.

In a preferred embodiment said mammalian cell or mammalian cell line is derived from human, horse, swine, goat, cattle, mouse or rat.

In a further preferred embodiment of the methods of the invention the cell or cell line is a chondrocyte, a fibroblast [97], a synovial cell, an endothelial cell, a macrophage, a tumour cell, a smooth muscle cell, a mesothelial cell, epithelial cell or a melanoma cell.

In a further embodiment of the methods of the invention, said cell is comprised in a tissue.

We analyzed the efficacy of several inhibitors in organ cultures of bovine articular cartilage that was induced to become osteoarthritic by interleukin. The inhibition of proteoglycan loss was analysed histologically by staining with safranin O. The following inhibitors were applied in different concentrations: Valspodar, Verapamil, Nicardipin, Nefidipine, Bepidril, Amiloride. Fig, 15 shows the conentration dependent inhibition of proteoglycan loss by valspodar. **Fig. 16** shows that valspodar and Verapamil gave the best protection. Also nicardipin and nefidipin that are similar to Verapamil showed fairly good protection, whereas bepidril and amyloride were only inhibitory at high concentrations. All 6 substances are ABC-B (MDR) inhibitors [53]. The results described above indicate that inhibitors of hyaluronan export from chondrocytes can be applied to protect from proteoglycan loss in arthritis.
Bovine articular cartilage can be obtained from a local slaughter house. Human carticular is obtained from therapeutic synovectomies. Inhibition of hyaluronan transport can be measured in slices of articular cartilage cultivated as organ cultures. Hyaluronan produced in cartilage slices can be visualized histochemically

by staining with labelled hyaluronan binding proteins such as the binding region of aggrecan or link protein.

Accordingly, in a preferred embodiment of the methods of the invention said tissue is cartilage tissue.

In a preferred embodiment of the methods of the present invention said cell or said tissue is derived from a mammalian subject preferably a human subject which suffers from arthritis. If this patient had a synovectomy or cartilage tissue was obtained during arthroscopy, the cartilage material can be taken into organ culture as described above and tested for recovery of cartilage production under the influence of the ABC transport inhibitors as described in the experimental section.

In another preferred embodiment of the methods of the present invention the cell comprises at least one heterologous ABC-transporter. In this context, the term "heterologous" means that the respective ABC-transporter (which is heterologous) is derived from another subject or has a different origin as the cell in question (e.g. expression of a bovine ABC-transporter in a human cell or expression of a human ABC-transporter in a bacterial/fungal/insect cell etc). Thus, it is envisaged that the cells to be used in the screening assay of the invention comprise a heterologous polynucleotide sequence which allows the expression of said heterologous ABC-transporter.

Preferably, the polynucleotide encoding the ABC-transporter is part of a vector, e.g. a commercially available vector. Nonlimiting examples include plasmid vectors compatible with mammalian cells, such as pUC, pBluescript (Stratagene), pET (Novagen), pREP (Invitrogen), pCRTopo (Invitrogen), pcDNA3 (Invitrogen), pCEP4 (Invitrogen), pMC1 neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2neo, pBPV-1, pdBPVMMTneo, pRSVgpt, pRSVneo, pSV2-dhfr, pUCTag, pIZD35, pLXIN and pSIR (Clontech) and pIRES-EGFP (Clontech). Baculovirus vectors such as pBlueBac, BacPacz Baculovirus Expression System (CLONTECH), and MaxBacTM Baculovirus Expression System, insect cells and protocols (Invitrogen) are available commercially and may also be used to produce high yields

of biologically active protein. (see also, Miller (1993), Curr. Op. Genet. Dev., 3, 9; O'Reilly, Baculovirus Expression Vectors: A Laboratory Manual, p. 127). In addition, prokaryotic vectors such as pcDNA2; and yeast vectors such as pYes2 are nonlimiting examples of other vectors suitable for use with the present invention. For vector modification techniques, see Sambrook and Russel (2001), loc. cit. Vectors can contain one or more replication and inheritance systems for cloning or expression, one or more markers for selection in the host, e. g., antibiotic resistance, and one or more expression cassettes.

The coding sequences inserted in the vector can be synthesized by standard methods, isolated from natural sources, or prepared as hybrids. Ligation of the coding sequences to transcriptional regulatory elements (e. g., promoters, enhancers, and/or insulators) and/or to other amino acid encoding sequences can be carried out using established methods.

Furthermore, the vectors may, in addition to the nucleic acid sequences of the invention, comprise expression control elements, allowing proper expression of the coding regions in suitable hosts. Such control elements are known to the artisan and may include a promoter, translation initiation codon, translation and insertion site or internal ribosomal entry sites (IRES) (Owens, Proc. Natl. Acad. Sci. USA 98 (2001), 1471-1476) for introducing an insert into the vector. Preferably, the nucleic acid molecule of the invention is operatively linked to said expression control sequences allowing expression in eukaryotic or prokaryotic cells. Particularly preferred are in this context control sequences which allow for correct expression in neuronal cells and/or cells derived from nervous tissue.

Control elements ensuring expression in eukaryotic and prokaryotic cells are well known to those skilled in the art. As mentioned above, they usually comprise regulatory sequences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally-associated or heterologous promoter regions. Possible regulatory elements permitting expression in for example mammalian host cells comprise the CMV-HSV thymidine kinase promoter, SV40, RSV-promoter (Rous sarcome virus), human elongation factor 1α-promoter, CMV enhancer, CaM-kinase promoter or SV40-enhancer.

For the expression in prokaryotic cells, a multitude of promoters including, for example, the tac-lac-promoter, the lacUV5 or the trp promoter, has been described. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pRc/CMV, pcDNA1, pcDNA3 (In-Vitrogene, as used, inter alia in the appended examples), pSPORT1 (GIBCO BRL) or pGEMHE (Promega), or prokaryotic expression vectors, such as lambda gt11. An expression vector according to this invention is at least capable of directing the replication, and preferably the expression, of the nucleic acids and protein of this invention. Suitable origins of replication include, for example, the Col E1, the SV40 viral and the M 13 origins of replication. Suitable promoters include, for example, the cytomegalovirus (CMV) promoter, the iacZ promoter, the gai10 promoter and the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) polyhedral promoter. Suitable termination sequences include, for example, the bovine growth hormone, SV40, iacZ and AcMNPV polyhedral polyadenylation signals. Examples of selectable markers include neomycin, ampicillin, and hygromycin resistance and the like. Specifically-designed vectors allow the shuttling of DNA between different host cells, such as bacteria-yeast, or bacteria-animal cells, or bacteria-fungal cells, or bacteria invertebrate cells.

A method for the production of a transgenic non-human animal, preferably transgenic mouse, is also within the scope of the present invention, said method comprising introduction of a polynucleotide or vector encoding one or more ABC-transporter(s) as described herein into a germ cell, an embryonic cell, stem cell or an egg or a cell derived therefrom. The non-human animal can be used in accordance with a screening method of the invention described herein and may be a non-transgenic healthy animal, or may have a degeneration and/or a destruction of cartilage, preferably by a disorder caused by an increased hyaluronan-transport across a lipid bilayer. Such transgenic animals are well suited for, e.g., pharmacological studies of drugs in connection with arthritis. Production of transgenic embryos and screening of those can be performed, e.g., as described by

A. L. Joyner Ed., Gene Targeting, A Practical Approach (1993), Oxford University Press. The DNA of the embryonal membranes of embryos can be analyzed using, e.g., Southern blots with an appropriate probe; see supra.

The present invention also opens up the possibility to produce e.g suitable test-systems like transgenic non-human animals with an increased level of the ABC-transporter(s) as described above and, thus, with an over-production/excess transport of hyaluronan across a lipid bilayer. Accordingly, it is possible to screen for compounds which subsequently reduce this over-expression of ABC-transporter(s) e.g. by the expression of antisense-RNA, ribozymes, of molecules which combine antisense and ribozyme functions and/or of molecules which provide for a co-suppression effect. When using the antisense approach for reduction of the amount of ABC-transporter(s) in cells, the nucleic acid molecule encoding the antisense-RNA is preferably of homologous origin with respect to the animal species used for transformation. However, it is also possible to use nucleic acid molecules which display a high degree of homology to endogenously occurring nucleic acid molecules encoding a ABC-transporter. In this case the homology is preferably higher than 80%, particularly higher than 90% and still more preferably higher than 95%. The reduction of the synthesis of a protein according to the invention in the transgenic mammalian cells can result in an alteration in, e.g., hyaluronan synthesis/transport across a lipid bilayer.

The effectiveness of a given antisense oligomer molecule in forming a heteroduplex with the target RNA may be determined by screening methods known in the art. For example, the oligomer is incubated in a cell culture expressing one or more ABC-transporter(s) as mentioned herein, and the effect on the target RNA is evaluated by monitoring the presence or absence of heteroduplex formation with the target sequence and non-target sequences using procedures known to those of skill in the art.

Accordingly, the present invention also relates to screening methods as indicated herein above, wherein the cell and/or said tissue is comprised in a non-human animal.

In this embodiment, the effect of the test compound may be assessed by observing the disease state of a non-human animal which is characterized by an increased hyaluronan transport as mediated by one or more ABC-transporter(s). Said increased hyaluronan transport may be mediated by the over-expression of one or more ABC-transporter(s) or by the stimulation of the transport of hyaluronan mediated by one or more ABC-transporter(s). With regard to the mentioned stimulation,it has to be understood that most growth factors and cytokines cause enhanced hyaluronan production in responsive target cells. Thus, if the animal suffers from an artificially induced arthritis (induced e.g. by over-expression or stimulation of one or more ABC-transporter(s)) prior to the administration of the test compound and the administration of the test compound (which may be repeated) results in an amelioration of the disease, then it can be concluded that the test compound is a prime candidate for the development of a medicament useful also in other mammalians, preferably humans. In addition, the compound can also inhibit the disease establishment when administered in advance.

In this context, it is envisaged that said non-human animal comprises a cell or a tissue comprising said cell as defined herein, which encodes and expresses at least one ABC-transporter which is capable of transporting hyaluronan across a lipid bilayer. Preferably, said cell or tissue comprising said cell is characterized by an over-expression of the ABC-transporter, i.e. the amount of the ABC-transporter in the cell is increased when compared with a reference cell (said over-expression can be easily quantified by methods well-known to the skilled person e.g. by antibody-binding; FACS-analysis; ELISA or the like). In one embodiment, it is envisaged that the over-expression is effected by transfecting the cell with a polynucleotide sequence which is capable of expressing one or more ABC-transporter(s) as defined herein. Corresponding methods for providing and transfecting a polynucleotide sequence encoding one or more ABC-transporter(s) are well-known to the skilled person. It is envisaged that the reference cell is for example a cell derived from the same subject but which was not transfected with a polynucleotide sequence capable of expressing one or more ABC-transporter(s).

In another embodiment, said over-expression of one or more ABC-transporter(s) can be effected by stimulating the expression and/or the hyaluronan transport capacity of one or more ABC-transporter(s) in order to simulate a disease state which can be correlated with arthritis.

Unlike spontaneous arthritis, experimental induction in animal makes it possible to influence the onset and course of the disease. The lesions can be studied macroscopically, histologically and biochemically, to assess the role of mediators such as cytokines, growth factors, and proteases. In the rat model spontaneous locomotor activity is rapidly, transiently, and dose-dependently decreased after iodoacetate injection into rat knees (primary response). Thereafter, only high doses (0.3 mg and 3.0 mg) lead to a secondary progressive long-term loss of spontaneous mobility on day 15, when subchondral bone is exposed. These 2 doses result in significant changes in cartilage proteoglycan concentration at day 15 and a strong inhibition of anabolism in the peripheral patellae by day 2, contrasting with the effects of lower doses (0.01, 0.03, and 0.1 mg). Thus when a sufficient dose of iodoacetate is used, this model can easily and quickly reproduce arthritis-like lesions and functional impairment in rats, similar to that observed in human disease. These parameters, as well as proteoglycan metabolism, serve as indicators for studying chondroprotective drugs, or for evaluating the ability of imaging techniques to detect and evaluate chondral lesions.

The rat model of osteoarthritis was utilized to test whether Verapamil could protect cartilage from the loss of proteoglycans [77]. Osteoarthritic damage was induced in the left knees iodoacetate into the synovial cavity. The right knees remained untreated and served as controls. Three rates were feed with normal drinking water and three with drinking water containing Verapamil. After 17 days the rats were sacrificed and the articular cartilage was analysed histologically. Acid polysaccharides were stained with alcian blue and the preparation was counterstained with nuclear fast red. **Fig. 17** shows that Verapamil completely inhibited proteoglcan loss. These results indicate that inhibitors of hyaluronan export from chondrocytes can be applied to protect from proteoglycan loss in arthritis.

It will be understood that some of the screening-methods of the present invention can be carried out *in vivo* or *ex vivo (in vitro)*.

The methods of the present invention also allows for the diagnosis of a subject at risk for arthritis or the diagnosis of arthritis, inter alia by the identification of an over-expression of ABC-transporter(s) and/or the identification of an increased transport of hyaluronan as mediated by ABC-transporter(s).

In accordance with this embodiment, the diagnosis can, e.g., be effected by isolating cells from an individual. Such cells can be collected from body fluids, skin, hair, biopsies and other sources. Collection and analysis of cells from bodily fluids such as blood, urine and cerebrospinal fluid is well known to the art; see for example, Rodak, "Haematology: Clinical Principles & Applications" second ed., WB Saunders Co, 2002; Brunzel, "Fundamentals of Urine and Body Fluids Analysis", WB Saunders Co, 1994; Herndon and Brumback (Ed.), "Cerebrospinal Fluid", Kluwer Academic Pub., 1989. In addition, methods for DNA isolation are well described in the art; see, for example, Sambrook et al., "Molecular Cloning: A Laboratory Manual", 3rd edition, Cold Spring Harbor Laboratory, 2001.

Thus, the present invention also relates to a method for identifying a subject at risk for arthritis comprising the steps of:

(a) analyzing the hyaluronan transport rate of a cell derived from said subject; and

(b) comparing the hyaluronan transport rate of said cell with the hyaluronan synthesis rate of a reference cell.

In a preferred embodiment said cell is a chondrocyte.

Over the past 20 years, genetic heterogeneity has been increasingly recognized as a significant source of variation in drug response. Many scientific communications (Meyer, Ann. Rev. Pharmacol. Toxicol. 37 (1997), 269-296 and West, J. Clin. Pharmacol. 37 (1997), 635-648) have clearly shown that some drugs work better or may even be highly toxic in some patients than in others and that these variations in

patient's responses to drugs can be related to molecular basis. This "pharmacogenomic" concept spots correlations between responses to drugs and genetic profiles of patient's (Marshall, Nature Biotechnology, 15 (1997), 954-957; Marshall, Nature Biotechnology, 15 (1997), 1249-1252).

In this context of population variability with regard to drug therapy, pharmacogenomics has been proposed as a tool useful in the identification and selection of patients which can respond to a particular drug without side effects. This identification/selection can be based upon molecular diagnosis of genetic polymorphisms by genotyping DNA from leukocytes in the blood of patient, for example, and characterization of disease (Bertz, Clin. Pharmacokinet. 32 (1997), 210-256; Engel, J. Chromatogra. B. Biomed. Appl. 678 (1996), 93-103). For the founders of health care, such as health maintenance organizations in the US and government public health services in many European countries, this pharmacogenomics approach can represent a way of both improving health care and reducing overheads because there is a large cost to unnecessary drugs, ineffective drugs and drugs with side effects.

In view of the disclosure content of the present invention, it will be understood that the methods of the present invention are also useful for monitoring the efficacy and/or dosing of a drug or the likelihood of a patient to respond to a drug (for example an inhibitor of the invention). Thus, in yet another embodiment the invention relates to a method for screening for a compound which is suitable for the treatment of arthritis in a subject, i.e. for monitoring the efficacy and/or dosing of a drug, e.g. an inhibitor as defined herein, and/or the likelihood of a patient to respond to said drug. Said method comprises contacting a cell derived from said subject which comprises at least one ABC-transporter (preferably an ABC-transporter which is able to transport hyaluronan) with a test-compound to be tested; determining/measuring the level of expression (either transcriptional or translational) of one or more ABC-transporter(s) and/or determining/measuring the level of hyaluronan transport mediated by ABC-transporter(s) across a lipid bilayer in said cell before and after administration of the respective drug. In humans, ABC-transporter activity can be monitored by Positron Emission Tomography (PET) or Single Photon Emission Computerised Tomography (SPECT) using a radiolabelled ligand tracer for ABC-transporter(s). A modulation of ABC-transporter activity, or

expression levels would reflect the activity and potency of the drug. Methods and techniques required for PET analysis are well known in the art, see, for example Paans and Vaalburg, Curr. Pharmac. Design 6 (2000), 1583-1591; van Waarde, Curr. Pharmac. Design. 6 (2000), 1593-1610; Paans et al, Methods 27 (2002), 195–207; Passchier et al., Methods 27 (2002), 278–286; Laruelle et al., Methods 27 (2002), 287–299.

Hence, the present invention also concerns the pharmacogenomic selection of drugs and prodrugs for patients suffering from arthritis and which are possible candidates to drug therapy. The findings of the present invention provide the options of development of new drugs for the pharmacological intervention with the aim of reducing and thereby normalizing the hyaluronan transport mediated by ABC-transporter(s) The terms "reducing" and "normalizing" have already been defined elsewhere in this specification. Also a gene therapeutical approach can be envisaged with the aid of the present invention.

The present invention relates to a method of screening for a compound which is suitable for the treatment of arthritis in a subject, said method comprising:

(a) contacting a cell derived from said subject which comprises at least one ABC-transporter with a test compound to be tested;

(b) measuring the effect of the test compound on the transport of an indicator compound across a lipid bilayer of said cell; and

(c) identifying compounds which reduce the transport of hyaluronan across the lipid bilayer of said cell.

In order to be suitable for the treatment of arthritis, it will be acknowledged that the compound identified has to be administered subsequently in a therapeutically effective dose which is also explained in other parts of the specification. A therapeutically effective dose refers to that amount of screened compounds/ inhibitors which ameliorate the symptoms or condition. Therapeutic efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50%

of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50.

In a preferred embodiment of the screening method for a compound which is suitable for the treatment of arthritis in a subject, said cell (which is derived from said subject) is a chondrocyte. The term "derived from a subject" as used herein means that at least one cell is isolated from said subject. Such cells can be collected from body fluids, skin, hair, biopsies and other sources. Collection and analysis of cells from bodily fluids such as blood, urine and cerebrospinal fluid is well known to the art and was already described herein above.

In another embodiment said cell is comprised in a tissue.

In another embodiment of the of screening method for a compound which is suitable for the treatment of arthritis in a subject, said subject is a mammalian subject.

In a preferred embodiment said mammalian subject is a human, a horse, a camel, a dog, a cat, a pig, a cow, a goat or a fowl.

In a further embodiment of the present invention said cell is contacted with a compound selected from the group consisting of:

(a) an inhibitor of a member of the ABCB (MDR)-subfamily selected from Verapamil, Valspodar (PSC833), Elacridar (GF-120918), Bericodar (VX-710), Tariquidar (XR-9576), XR-9051, S-9788, LY-335979, MS 209, , R101933; OC-144-093; Quinidine, Chloripramine, Nicardipine, Nifedipine, Amlodipine, Felodipine, Manidipine, Flunarizine, Nimodipine, Pimozide, Lomerizine, Bepridil, Amiloride, Almitrine, Amiodarone, Imipramine, Clomiphene, Tamoxifen, Toremifene, Ketocanazole, Terfenadine, Chloroquine, Mepacrin, Diltiazem, Niguldipine, Prenylamine, Gallopamil, Tiapamil, Dex-Verapamil, Dipyridamole, Pimozide, Haloperidol, Chlorpromazine, Trifluoperazine, Fluphenazine, Reserpin, Clopenthixol, Flupentixol, N-acetyldaunorubicin, Vindoline, N2762-14, N276-14, N276-17, B9309-068, BIBW-22, Carvedilol, Clofazimine, Ketoconazole, Lovastatin, N-Norgallopamil, Simvastatin,

Troleandomycin, Vinblastin, Itraconazole, Econazole, Oligomycine, Cyclosporin and Rapamycin; and/or

(b) an inhibitor of a member of the ABCA subfamily selected from Glyburide, DIDS (4,4-diisothiocyanatostilbene-2,2-disulfonic acid), Bumetanide, Furosemide, Sulfobromophthalein, Diphenylamine-2-carboxylic acid and Flufenamic acid; and/or

(c) an inhibitor of a member of the human ABC-C (MRP)-subfamily selected from MK-571, Benzbromaron, PAK-104P, Probenecid, Sulfinpyrazone, Indomethacin, Merthiolate and Ethacrynic acid; and/or

(d) (an) antibody(ies) or functional fragments thereof which is(are) specifically recognizing one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer; and/or

(e) (an) antisense oligomere(s), iRNA and/or siRNA directed against one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer; and/or

(f) (an) aptamer(s) directed against one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer.

The test-compound is in a further embodiment of the screening-methods of the present invention a small molecule or a peptide derived from an at least partially randomised peptide or aptamer library.

Furthermore, the present invention relates to methods which further comprise a step of refining the compound identified, said method comprising the steps of:

(a) identification of the binding sites of the compound and the ABC-transporter(s);

(b) molecular modelling of the binding site of the compound; and

(c) modification of the compound to improve its binding specificity for the ABC-transporter(s).

All techniques employed in the various steps of the method of the invention are conventional or can be derived by the person skilled in the art from conventional techniques without further ado. Thus, biological assays based on the herein

identified nature of the polypeptides may be employed to assess the specificity or potency of the *inhibitors* (sometimes also referred to as *drugs*) wherein the increase or decrease, e.g of the hyaluronan-transport across a lipid bilayer, may be used to monitor said specificity or potency. Steps (a) and (b) can be carried out according to conventional protocols. A protocol for site directed mutagenesis is described in Ling MM, Robinson BH. (1997) Anal. Biochem. 254: 157-178. The use of homology modeling in conjunction with site-directed mutagenesis for analysis of structure-function relationships is reviewed in Szklarz and Halpert (1997) Life Sci. 61:2507-2520. For example, identification of the binding site of said drug by site-directed mutagenesis can be achieved by modifications in the primary sequence of an identified ABC-transporter which is responsible for an excess transport of hyaluronan, that affect the drug affinity; this usually allows to precisely map the binding pocket for the drug.

As regards step (b), the following protocols may be envisaged: Once the effector site for drugs has been mapped, the precise residues interacting with different parts of the drug can be identified by combination of the information obtained from mutagenesis studies (step (a)) and computer simulations of the structure of the binding site provided that the precise three-dimensional structure of the drug is known (if not, it can be predicted by computational simulation). If said drug is itself a peptide, it can be also mutated to determine which residues interact with other residues in the polypeptide of interest.

Finally, in step (c) the drug can be modified to improve its binding affinity or ist potency and specificity. If, for instance, there are electrostatic interactions between a particular residue of interest and some region of the drug molecule, the overall charge in that region can be modified to increase that particular interaction.

Identification of binding sites may be assisted by computer programs. Thus, appropriate computer programs can be used for the identification of interactive sites of a putative inhibitor and the polypeptide by computer assisted searches for complementary structural motifs (Fassina, Immunomethods 5 (1994), 114-120). Further appropriate computer systems for the computer aided design of protein and peptides are described in the prior art, for example, in Berry, Biochem. Soc. Trans. 22 (1994), 1033-1036; Wodak, Ann. N. Y. Acad. Sci. 501 (1987), 1-13; Pabo, Biochemistry 25 (1986), 5987-5991. Modifications of the drug can be produced, for

example, by peptidomimetics and other inhibitors can also be identified by the synthesis of peptidomimetic combinatorial libraries through successive chemical modification and testing the resulting compounds. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh, Methods in Enzymology 267 (1996), 220-234 and Dorner, Bioorg. Med. Chem. 4 (1996), 709-715.

In accordance with the above, in a preferred embodiment of the method of the invention said compound is further refined by peptidomimetics.

The invention furthermore relates in a further preferred embodiment to a method of modifying a compound identified or refined by the method as described herein above as a lead compound to achieve (i) modified site of action, spectrum of activity, organ specificity, and/or (ii) improved potency, and/or (iii) decreased toxicity (improved therapeutic index), and/or (iv) decreased side effects, and/or (v) modified onset of therapeutic action, duration of effect, and/or (vi) modified pharmakinetic parameters (resorption, distribution, metabolism and excretion), and/or (vii) modified physico-chemical parameters (solubility, hygroscopicity, color, taste, odor, stability, state), and/or (viii) improved general specificity, organ/tissue specificity, and/or (ix) optimized application form and route by (i) esterification of carboxyl groups, or (ii) esterification of hydroxyl groups with carbon acids, or (iii) esterification of hydroxyl groups to, e.g. phosphates, pyrophosphates or sulfates or hemi succinates, or (iv) formation of pharmaceutically acceptable salts, or (v) formation of pharmaceutically acceptable complexes, or (vi) synthesis of pharmacologically active polymers, or (vii) introduction of hydrophylic moieties, or (viii) introduction/exchange of substituents on aromates or side chains, change of substituent pattern, or (ix) modification by introduction of isosteric or bioisosteric moieties, or (x) synthesis of homologous compounds, or (xi) introduction of branched side chains, or (xii) conversion of alkyl substituents to cyclic analogues, or (xiii) derivatisation of hydroxyl group to ketales, acetales, or (xiv) N-acetylation to amides, phenylcarbamates, or (xv) synthesis of Mannich bases, imines, or (xvi) transformation of ketones or aldehydes to Schiff's bases, oximes, acetales, ketales, enolesters, oxazolidines, thiozolidines or combinations thereof.

The various steps recited above are generally known in the art. They include or rely on quantitative structure-action relationship (QSAR) analyses (Kubinyi, "Hausch-Analysis and Related Approaches", VCH Verlag, Weinheim, 1992), combinatorial biochemistry, classical chemistry and others (see, for example, Holzgrabe and Bechtold, Deutsche Apotheker Zeitung 140(8), 813-823, 2000).

The invention moreover relates in a further preferred embodiment to a method further comprising producing a pharmaceutical composition comprising formulating the compound identified, refined or modified by the method of any of the preceding claims with a pharmaceutically active carrier and/or diluent.

Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. A typical dose can be, for example, in the range of 0.001 to 1000 µg (or of nucleic acid for expression or for inhibition of expression in this range); however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{12} copies of the DNA molecule. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery. Preparations for parenteral

administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents such as interleukins or interferons depending on the intended use.

The present invention also relates to a method for manufacturing a pharmaceutical composition comprising the steps of any one of the aforementioned screening methods and the step of formulating the compound screened in a pharmaceutically acceptable form. In this regard, the term "in a pharmaceutically acceptable form" refers to the formulation of the compounds screened with a pharmaceutically acceptable carrier and/or diluent. Examples of suitable pharmaceutical carriers are well known in the art and have already been described herein above.

The present invention also relates to a method of preventing, ameliorating and/or treating the symptoms of arthritis in a subject comprising administering at least one inhibitor as defined herein /screened by the methods disclosed herein of at least one ABC-transporter capable of transporting hyaluronan across a lipid bilayer to the subject such that the arthritis is prevented, ameliorated and/or treated.

The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of partially or completely curing a disease and/or adverse effect attributed to the disease. The term "treatment" as used herein covers any treatment of a disease in a mammal, particularly a human,

and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e. arresting its development; or (c) relieving the disease, i.e. causing regression of the disease. The present invention is directed towards treating patients with medical conditions relating to arthritis. Accordingly, a treatment of the invention would involve preventing, inhibiting or relieving any medical condition related to arthritis. As mentioned elsewhere before, said arthritis is e.g. characterized by a degeneration and/or a destruction of cartilage.

In a preferred embodiment said arthritis is osteoarthritis, (juvenile) chronic arthritis, rheumatoid arthritis, psoriatic arthritis, *A. mutilans*, septic arthritis, infectious arthritis and/or reactive arthritis.

In the context of the present invention the term "subject" means an individual in need of a treatment of an affective disorder. Preferably, the subject is a mammalian, particularly preferred a human, a horse, a camel, a dog, a cat, a pig, a cow, a goat or a fowl.

The term "administered" means administration of a therapeutically effective dose of the inhibitors and/or test-compounds as disclosed herein. By "therapeutically effective amount" is meant a dose that produces the effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques.

The methods are applicable to both human therapy and veterinary applications. The compounds described herein having the desired therapeutic activity may be administered in a physiologically acceptable carrier to a patient, as described herein. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways as discussed below. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt %. The agents maybe administered alone or in combination with other treatments, i.e. it is also within the scope of the present invention to combine for example one of

the already known drugs/treatments for arthritis (e.g. the injection of hyaluronan) with one or more of the inhibitors/test-compounds as defined herein.

The administration of the pharmaceutical composition can be done in a variety of ways as discussed above, including, but not limited to, orally, subcutaneously, intravenously, intra-arterial, intranodal, intramedullary, intrathecal, intraventricular, intranasally, intrabronchial, transdermally, intranodally, intrarectally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, or intraocularly. In some instances, for example, in the treatment of wounds and inflammation, the candidate agents may be directly applied as a solution dry spray.

It is also envisaged that the inhibitors/test-compounds of the present invention are employed in co-therapy approaches, i.e. in co-administration with other medicaments or drugs, for example other drugs for preventing, treating or ameliorating arthritis.

Another aspect of the present invention is the administration of an inhibitor which leads to a reduction of the expression of a nucleic acid encoding an ATP-transporter (as defined herein) in the cells or comprising a nucleic acid molecule the expression of which in cells or the administration of which to cells leads to a reduction of the expression of a nucleic acid encoding an ATP-transporter (as defined herein) in the cells. Said inhibitor may be useful for treating individuals having an increased amount of the ABC-transporter or expression level as described hereinabove.

Preferably, the above-mentioned inhibitor is an antisense, a ribozyme, a co-suppressive nucleic acid, iRNA or siRNA.

An siRNA approach is, for example, dislosed in Elbashir ((2001), Nature 411, 494-498)). It is also envisaged in accordance with this invention that for example short hairpin RNAs (shRNAs) are employed in accordance with this invention as pharmaceutical composition. The shRNA approach for gene silencing is well known in the art and may comprise the use of st (small temporal) RNAs; see, inter alia, Paddison (2002) Genes Dev. 16, 948-958.

As mentioned above, approaches for gene silencing are known in the art and comprise "RNA"-approaches like RNAi or siRNA. Successful use of such

approaches has been shown in Paddison (2002) loc. cit., Elbashir (2002) Methods 26, 199-213; Novina (2002) Mat. Med. June 3, 2002; Donze (2002) Nucl. Acids Res. 30, e46; Paul (2002) Nat. Biotech 20, 505-508; Lee (2002) Nat. Biotech. 20, 500-505; Miyagashi (2002) Nat. Biotech. 20, 497-500; Yu (2002) PNAS 99, 6047-6052 or Brummelkamp (2002), Science 296, 550-553. These approaches may be vector-based, e.g. the pSUPER vector, or RNA polIII vectors may be employed as illustrated, inter alia, in Yu (2002) loc. cit.; Miyagishi (2002) loc. cit. or Brummelkamp (2002) loc. cit.

A compound which leads to a reduction of the expression of an ABC-transporter gene in question may, e.g., be a compound which acts on the regulatory region of the gene and thereby reduces the level of transcription. Such compounds can be identified by methods as described herein.

This disclosure may best be understood in conjunction with the accompanying drawings, incorporated herein by references. Furthermore, a better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration and are not intended as limiting.

The figures show:

Fig. 1 Classification of human ABC transporters and their inhibitors.

Fig. 2 Kinetics of hyaluronan production by intact streptococci: Group A *Streptococcus pyogenes* M49 (strain CS101pGhost9:IS*S1*) ( ), the ABC transporter mutant (~) and the rescued transfectant (•) were grown to the exponential growth phase, harvested and incubated with [^{3}H]glucosamine for determination of hyaluronan synthesis in intact The amount of total [^{3}H]hyaluronan in the culture supernatant was determined at the times indicated.

Fig. 3 Hyaluronan synthase activity and capsule production.

Fig. 4 Arrangement of genes for hyaluronan synthesis and export:
This chromosomal segment from the known sequence of *Streptococcus pyogenes* M1 (strain SF370) from the data base at the University of Oklahoma Advanced Center for Genome Technology covers the loci for hyaluronan synthesis (*hasA*, *hasB* and *hasC*) and for the ABC transporter (*haxA*, *haxB*, *haxC* and *haxD*). IS*S1*, insertion sequence; Erm, erythromycin resistance; primer 1, haxupSacI; primer 2, haxdownPstI; primer 3, pGhost5SK; primer 4, pGhost5KS; primer 5, ISpGhost9P7; primer 6, ISpGhost9P8.

Fig. 5 Phylogenetic relationship of the streptococcal HaxA and HaxB proteins with human ABC transporter.

Fig. 6 Concentration dependent inhibition of growth and hyaluronan production of human skin fibroblasts by Verapamil and Valspodar.

Fig. 7 Concentration dependent inhibition of growth and hyaluronan production of human synovial fibroblasts by Verapamil and Valspodar.

Fig. 8 Concentration dependent inhibition of hyaluronan synthase activity in membranes form human skin fibroblasts by Verapamil and Valspodar.

Fig. 9 Concentration dependent inhibition of hyaluronan production of the human colon carcinoma cell lines HT29 and HT29 mdr by Verapamil.

Fig. 10 Concentration dependent inhibition of hyaluronan synthase activity of membranes from the human colon carcinoma cell lines HT29 and HT29 mdr by Verapamil.

Fig. 11 Concentration dependent inhibition of hyaluronan production of a human fibroblast cell line by glyburide and Valspodar.

Fig. 12 Concentration dependent inhibition of hyaluronan synthase activity in membranes from a human fibroblast cell line by valspodar, benzbromarone and MK-571.

Fig. 13 Concentration dependent inhibition of hyaluronan production of a human chondrocyte cell line by Valspodar.

Fig. 14 Effect of valspodar on proteoglycan production of a human chondrocyte cell line.

Fig. 15 Concentration dependent inhibition of proteoglycan loss from osteoarthritic bovine cartilage by Valspodar.

Fig. 16 Protection from proteoglycan loss from osteoarthritic cartilage in rat knee joints.

Fig. 17 Inhibition of proteoglycan loss from osteoarthritic bovine cartilage by inhibitors of ABC-transporters.

Examples:

The following examples illustrate the invention. These examples should not be construed as to limit the scope of this invention. The examples are included for purposes of illustration and the present invention is limited only by the claims.

Cells and cell culture

Fibroblasts were grown in suspension culture in Dulbecco's modified Eagles medium supplemented with streptomycin/penicillin (100 units of each/ml), kanamycin (100 units/ml) and 10 % foetal calf serum. The human colon carcinoma cell lines HT29 and HT29-mdr were from Dr. U. Schumacher (Universitätsklinikum Hamburg) [72]. Synovial fibroblasts were extracts from synovial membranes obtained from therapeutic synovectomies. A temperature sensitive human chondrocyte cell line (tsT/AC62) was obtained from Dr. M. Goldring, Boston (REF).

General Methods

Isolation of membranes and the determination of hyaluronan synthase activity was performed as described previously [86]. The concentration of hyaluronan in the cell culture medium was determined by an ELISA [74;87]. The proteoglycan concentration was measured by a colour reaction [75], and sulfate incorporation into proteoglycans was determined by radioactivity [76].

Example 1: Hyaluronan synthesis and export in Streptococci

Materials

Dry media (Todd-Hewitt, TH) and media components were from GIBCO BRL. Sheep blood was from Oxoid. [^{3}H]GlcN (specific activity 18.5 Ci/mMole) was from Amersham International, UDP-[^{14}C]GlcA (specific activity 0.3 Ci/mMole) and UDP-[^{3}H]GlcNac (specific activity 34.8 Ci/mMol) were from NEN. The DEAD/LIVE BacLight Bacterial Viability Kit was from Molecular Probes, Inc. All other chemicals were purchased from Sigma except stated otherwise. Restriction and other DNA modifying enzymes were from New England Biolabs. Oligonucleotide primers were synthesized by MWG Biotech.

Bacterial strains and plasmids

Group A *Streptococcus pyogenes* M49 (strain CS101) was from A.Podbielski. Thermosensitive pGhost9:IS*S1* vector containing the IS*S1* insertion sequence from *Lactococcus lactis* and an erythromycin resistance marker was obtained from E. Maguin [64]. *E. coli* EC101 was provided by K. Leenhout [78]. pAT28 vector with a spectinomycin resistance marker was available from P. Courvalin [79].

General methods

Streptococci were grown on Todd-Hewitt agar supplemented with 3% sheep blood (Oxoid), in Todd-Hewitt medium (TH) or in TH supplemented with 0,5% yeast (THY) at 37°C. CS101 mutant strain containing pGhost9:IS*S1* vector was subcultured in medium supplemented with erythromycin (5 mg/l) at 37°C. Standard recombinant DNA techniques for nucleic acid preparation and analysis were performed as described [80]. DNA restriction fragments were isolated from agarose gels with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Electrotransformation of *E. coli* was performed by the method of Dower et al. [81] with a BioRad Gene Pulser (BioRad Laboratories). Genomic streptococcal DNA was isolated as described previously [82]. Alternatively to phenol/chloroform extraction, DNeasy Tissue Kit (Qiagen) was employed after treating the cells with pronase. Plasmids were sequenced on an ABI 310 automated DNA sequencer using the ABI PRISM® Big Dye terminator cycle sequencing kit (PE Applied Biosystems).

Electroporation of streptococci

Streptococci were transformed by electroporation as described [83] with some modifications. Briefly, 5 ml overnight culture in THY supplemented with 20 mM glycin and 5% serum was diluted 20-fold in the same medium and grown to an OD_{600} of 0,3. After addition of 20 mg hyaluronidase the bacteria were incubated at 37°C for further 15 min and cooled on ice for an hour. The cells were harvested by centrifugation at 2,000g and 4°C for 10 min and washed twice in cold electroporation buffer (EPM, 272 mM glucose, 1mM $MgCl_2$, pH 6,5/NaOH) and finally resuspended in 0,5 ml of cold EPM. 2 µg plasmid DNA was mixed with 100 µl of the cell suspension and electroporation was carried out at 1400 V, 25 µF and 200 Ω in a Gene Pulser (Bio-Rad Laboratories). The cells were quickly transferred into 5 ml THY supplemented with 5% serum, incubated for 2 to 3 hours at 37°C and plated onto selective agar plates.

Construction of S. pyogenes mutant library

The *Streptococcus pyogenes* mutant library was constructed by chromosomal insertion of the thermosensitive pGhost9:IS*S1* vector [64]. Bacteria were transformed by electroporation with 1 µg of purified plasmid DNA. Selection of plasmid containing strains was performed on blood agar plates supplemented with erythromycin (5mg/l) at a temperature of 30°C, allowing replication of the vector. To generate chromosomal integration of the plasmid, one of the isolates was grown overnight in THB (Todd Hewitt Broth, Oxoid) supplemented with erythromycin (5 mg/l) at 30°C. The saturated culture was diluted 1:100 in fresh THB medium without antibiotic pressure and incubated for 3 h at 30°C. To reduce the plasmid copy number per bacterial cell, the culture was transferred to a 38°C water bath and incubated for another 2 h. Samples were diluted with fresh THB medium and plated on erythromycin containing blood agar plates (1 mg/l). Mutants were selected for further studies after overnight growth at nonpermissive temperature (above 37°C).

Determination of the ISS1 insertion site

To identify the chromosomal insertion site of the IS*S1* insertion sequence, 2 µg of total genomic DNA of the pGhost9:IS*S1* mutants was digested with *Eco*RI or *Hind*III,

extracted with phenol/chloroform and ethanol precipitated. The digested DNA was diluted to a final concentration of 0.5 µg/ml and ligated with the T4 DNA ligase under conditions as described [84].The obtained plasmid mixture was transfected into *E. coli* EC101 [78]. Erythromycin-resistant *E. coli* clones containing pGhost9:IS*S1* plasmid with genomic streptococcal DNA flanking the insertion site of the plasmid were selected on LB agar with erythromycin (150 mg/l). The genomic DNA contained in the recovered plasmids was sequenced with primers annealing to pGhost9:IS*S1* vector sequences. To sequence the plasmids obtained after EcoRI digestion we used primers pGhost5SK and ISpGhost9P8. Primers pGhost5KS and ISpGhost9P7 were used to sequence plasmids obtained after HindIII digestion. The location of the primers is demonstrated in Fig. 2 and their sequences are indicated in Table 1.

Cloning of the hax locus

For complementation studies we cloned the *hax* locus into pAT28 plasmid by PCR of the genomic DNA of the wild type strain with primers haxupSacI and haxdownPstI (for primer sequences and locations see Table 1 and Fig. 2) and subsequent restriction digestion with SacI and PstI and ligation into the pAT28 plasmid. After amplification in *E. coli* the construct was electroporated into *S. pyogenes*. Successful transfer of the plasmid was confirmed by plasmid preparation and subsequent PCR amplification of the cloned fragment with the primers that were used to clone the *hax* locus.

Determination of bacterial hyaluronan synthase activity

Overnight cultures of streptococci were diluted at a ratio of 1:10 with fresh media and incubated at 37°C until an exponential growth phase. The bacteria (10 ml) were sedimented at 2,000g for 10 min, washed twice with 50 mM Tris-malonate (pH 7,0) and suspended in 2 ml 50 mM Tris-malonate (pH 7,0), 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.15 M NaCl. The cells were lysed by ultrasonication and ultracentrifuged at 50,000g and 4°C for 15 min. The membrane pellet was resuspended in 250 µl of the same buffer by short ultrasonication. To 50 µl of the membrane suspension 25 µl of the substrate for hyaluronan synthesis (160 _M UDP-GlcNac and 8 _M UDP-[^{14}C]GlcA (specific activity 320 mCi/mmol, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.15 M

NaCl) and incubated for 1 h at 37°C. A solution (10 µl) of 10 % SDS was added to inactivate the synthase and the mixture was applied to descending paper chromatography on Whatman MM paper for 18 h in 1 M ammonium acetate, pH 5.5/ethanol (13:7). The origin was cut out and radioactivity determined.

Measurement of hyaluronan release by intact bacteria

The rate of hyaluronan synthesis by intact bacteria was measured by incorporation of radioactivity into hyaluronan using [^{3}H]glucosamine in the presence of excess non-radioactive UDP-N-acetyl-glucosamine. Overnight cultures of *Streptococcus pyogenes* were diluted at a ratio of 1:10 with fresh media and incubated at 37°C for three hours to reach exponential growth. Aliquots of 1 ml were sedimented for 1 min at 10,000 g, suspended in 0.5 ml of fresh medium, mixed with 10 µl of a 100 µg/µl solution of UDP-GlcNac and 25 µl of [^{3}H]glucosamine and incubated at 37°C. After different time periods the suspension was again centrifuged for 1 min at 10,000 g. The supernatant was subjected to the descending paper chromatography as described above.

Determination of the hyaluronan capsule

The capsule of the streptococcal cells was determined by the method of Schrager et al. [85] with slight modifications. Cells from exponentially growing streptococci were wasched twice with water and suspended in 0.5 ml of water. The capsule was released by shaking with 1 ml of chloroform. After centrifugation the hyaluronan content in the water phase was determined by addition of 2 ml of a solution of 20 mg of stains-all (1-ethyl-2-[1-ethylnaphto-[1,2-d]thiazolin-2-ylidene)-2-methylpropenol]naphto-[1,2-d]thiazolium bromide) and 60 µl of acetic acid in 100 ml of dimethylformide. The absorbance was read at 640 nm and compared to a standard curve of known hyaluronan concentrations.

Bacterial vital stain

Dead and viable cells were differentiated by a DEAD/LIVE BacLight Bacterial Viability Kit. Shortly, 2 ml of a streptococcal culture was centrifuged and washed twice with 50 mM Tris-HCl, 0.9% NaCl pH 7.3 (TBS). The final pellet was resuspended in 3 ml TBS. The suspension (50 µl) was mixed with 1,5 µl staining

solution and incubated for 15 min at room temperature in the dark. The staining solution was prepared by mixing 1 µl 1,67 mM SYTO9, 1 µl 20 mM propidium iodide and 98 µl water. Fluorescence spectroscopy of the samples was carried out according to the manufacturer's instructions.

Example 2: Determination of the effect of ABC transport inhibitors on cell proliferation and hyaluronan production

Cells were seeded in 24 multiwell dished with a diameter of 2 cm^2 at a density of $2x10^4$ cells/cm^2 in Dulbecco's media containing 10% foetal bovine serum and increasing concentrations of inhibitors. After incubation for 3 days at 37°C the cells were trypsinized and counted. The concentration of hyaluronan was determined in the culture medium by an ELISA assay [87].

Example 3: Determination of the effect of ABC transport inhibitors on the activity of the hyaluronan synthase in membranes

For determination of the concentration dependent inhibition of inhibitors on the hyaluronan synthase, cells were grown in stationary flasks (180 cm^2) to near confluency. Four hours before harvest the cells were again stimulated by addition of 5% foetal bovine serum, washed with cold PBS and scrapped off with the aid of a rubber policemen. The cell suspension was collected in 30 ml of PBS and disrupted by nitrogen cavitation in a Parr bomb (15 min at 900 psi). The particular fraction was sedimented by ultracentrifugation (20 min at 100.000 g). The sediment was suspended in 1 ml of a solution of 160 µM UDP-GlcNac and 8 µM UDP-[^{14}C]GlcA (specific activity 320 mCi/mmol, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.15 M NaCl. Aliquots (100 µl) were supplemented with increasing concentrations of inhibitors and incubated for 4 hours at 37°C. The hyaluronan synthase was inactivated by addition of 10 µl 0f 10% SDS. The suspension was subjected to descending paper

chromatography for 20 hours on Whatman MM in 1 M ammonium acetate, pH 5.5/ethanol (13:7). The origin was cut out and their radioactivity radioactivity was determined.

Example 4: Inhibition of haluronan synthesis by valspodar in human chondrocytes

Human chondrocytes were grown in RPMI media at 32°C to near confluency and harvested by trypsinization as described [73].The cells were suspended in an alginate solution (1.2% in 0.9% NaCl) at a cell density of $4x10^6$ cells/ml and pressed though a 22G syringe dropwise (3 drops) into a solution of 200 µl of 55 mM Na-citrate in 0.9% NaCl, pH 6.05 that had been added in the wells of a microtiter plate. This treatment leads to the formation of alginate beads containing chondrocytes. The beads in the wells of the microtiter plate were washed with PBS and incubated with RPMI-media for 5 days at 39°C. The media were than replaced with fresh media with and without interleukin-1ß (200 pg/ml) and increasing concentrations of valspodar and incubated for another 3 days at 39°C. The media were withdrawn, centriguded for 5 min at 2000 g and frozen until the hyaluronan assay was performed. The beads were washed with PBS and solubilzed with a solution of 125 µl 55 mM Na-citrate in 0.9% NaCl, pH 6.05 for 10 min at 37°C. The cells were sedimented at 2000 g for 5 min and suspended in 125 µl of a solution of 20 µg/ml Papain in 0.1 M Na-acetat pH 5.53, 50 mM EDTA, 0.9% NaCl, 5 mM cysteine and incubated for 20 hours at 37°C. The supernatants were supplemented with 12.5 µl of 200 µg/ml papain in 0.1 M Na-acetat pH 5.53, 50 mM EDTA, 0.9% NaCl, 5 mM cysteine and also incubated for 20 hours at 37°C. Aliquotes of these solutions were taken for determination of the hyaluronan concentration by an Elisa assay.

Example 5: Effect of valspodar on proteoglycan synthesis in human chondrocytes

Human chondrocyten were grown as described above. Aliquotes were taken for the determination of the proteoglycan concentration using a colour reaction as described [75]. For measurement of the proteoglycan synthesis rate the chondrocytes in the wells of the microtiter plates were supplemented with 12.5 µl $[^{35}S]SO4^{2-}$ (0.5 mCi/ml) 20 hours before harvest. Aliquotes (20 µl) were used for the determination of radioactivity incorporated into $[^{35}S]$Proteoglycans as described [76].

Example 6: Determination of the inhibitory effects of drugs on proteoglycan loss osteoarthritic bovine cartilage

A bovine knee was obtained from a local slaughter and slices of cartilage (0.5 cm^2) were were incubated in 1 ml of RPMI media containing 10% of foetal bovine serum in the presence and absence of interleukin-1ß (5 ng/ml) and increasing concentrations of valspodar for 3 days at 37°C. The tissues were stained histologically with fast green and safranin O. This stains proteoglycans red and other material greyish green.

Example 7: Treatment of osteoarthritic rats with Verapamil

The animal trials were performed in accordance with the guidelines of the local ethical committee. Osteoarthritic damage was induced in the left knees of 6 Wistar rats by injection of a solution of 50 µl of 100 mM iodoacetate into the synovial cavity. The right knees remained untreated and served as controls. Three rates were feed with normal drinking water and three with drinking water containing 0.75 mg/ml of Verapamil. This concentration has been used previously [88]. After 17 days the rats were sacrificed and the articular cartilage was analysed histologically. Acid polysaccharides were stained with alcian blue and the preparation was counterstained with nuclear fast red.

Example 8: Specific screening assay for the hyaluronan transporter

The specific screening assay for the hyaluronan transporter is based on the extrusion of labelled hyaluronan oligosaccharides from intact cells in monolayer culture. For this assay the labelled oligosaccharides have to be introduced into the cytosol of cells. Because they will normally not transverse the plasma membranes, they are introduced by osmotic lysis of pinocytotic vesicles according to a method that has already successfully been applied for the introduction of periodate oxidized nucleotide sugars [25]. Alternatively, they can be introduced with the aid of cationic lipid formulations such as lipofectamine or lipofectin according to procedures that are widely used to introduce nucleic acids into living cells [116].

Experimental

Hyaluronan oligosaccharides are prepared from commercially available hyaluronan by digestion with hyaluronidase and sized fractionation by gel filtration as described [102]. Appropriate oligosaccharide fractions having a lenght between 2 and 50 disaccharide units are labelled by incorporation of a biotin, radioactivity, or a fluorescent probe. These methods are routine published procedures [87,99-101,103].The cells are seeded into multiwell microtiter plates to a density of at least 4x104 cells/cm^2. When the cells are attached to the plastic surface after a few hours, they are washed with phosphate buffered saline and incubated with the labelled hyaluronan dissolved in medium for osmotic lysis of inocytotic vesicles (growth medium such as Dulbeccos medium containing 1 M sucrose, 50% poly(ethylene glycol)-1000) for at least 5 min up to several hours at 37°C. During this time the cells will pinocytose this hyperosmotic medium and the labelled hyaluronan. The above medium is substituted by a mixture of Dulbeccos medium and water (3:2) for 2 min. This causes the intracellular pinocytotic vesicles to lyse and to liberate the contents into the cytosol without damaging the cells. The cells can be subjected to this incubation sequence several times. The cells are washed thoroughly several times with phosphate buffered saline or growth medium to remove extracellular labelled hyaluronan and are then ready for the assay. They are incubated in growth medium containing the compound to be tested in different

concentrations for several hours. During this time the labelled hyaluronan will be transported back into the medium. The amount of labelled hyaluronan oligosaccharide in the medium can be determined by a biotin-related assay, by radioactivity or be fluorescence intensity.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other disclosures) in the Background of the Invention, Detailed Description, and Examples is hereby incorporated herein by reference.

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inbronchoalveolar lavage fluid from breast cancer patients issuppressed by smoking. Eur. Respir. J., 5, 785-790.

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[133] Tufveson,G., Hallgren,R., Johnsson,C., & Wahlberg,J. Use ofhyaluronidase in treatment of interstitial edema from organ grafts. Tufveson, Gunnar, Hallgren, Roger, Johnsson, Cecilia, and Wahlberg, Jan. WO 97-SE1313[WO 9808538 A1]. 1998. PCT Int. Appl., 18 pp. CODEN: PIXXD2. 24-7-1997.
Ref Type: Patent

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Claims

1. Use of at least one inhibitor of at least one ABC-transporter capable of transporting hyaluronan across a lipid bilayer, for the preparation of a pharmaceutical composition for the treatment of arthritis.

2. The use of claim 1, wherein said inhibitor(s) specifically reduce(s) the transport of hyaluronan across a lipid bilayer mediated by at least one of said ABC-transporter(s).

3. The use of claims 1 or 2, wherein said ABC-transporter(s) is(are) a mammalian ABC-transporter(s).

4. The use of any one of claims 1 to 3, wherein said ABC-transporter(s) is(are) a human ABC-transporter(s).

5. The use of any one of claims 1 to 4, wherein said human ABC-transporter(s) is(are) a member of the human ABCB (MDR)-subfamily, the ABCA subfamily and/or the human ABC-C (MRP)-subfamily.

6. The use of any one of claims 1 to 5, wherein said ABC-transporter(s) is(are) comprised in a chondrocyte cell, preferably a human chondrocyte cell.

7. The use of any one of claims 1 to 6, wherein said inhibitor(s) is(are) selected from the group consisting of:

   (a) an inhibitor of a member of the ABCB (MDR)-subfamily selected from Verapamil, Valspodar (PSC833), Elacridar (GF-120918), Bericodar (VX-710), Tariquidar (XR-9576), XR-9051, S-9788, LY-335979, MS 209, R101933; OC-144-093; Quinidine, Chloripramine, Nicardipine, Nifedipine, Amlodipine, Felodipine, Manidipine, Flunarizine, Nimodipine, Pimozide, Lomerizine, Bepridil, Amiloride, Almitrine, Amiodarone, Imipramine, Clomiphene, Tamoxifen, Toremifene,

Ketocanazole, Terfenadine, Chloroquine, Mepacrin, Diltiazem, Niguldipine, Prenylamine, Gallopamil, Tiapamil, Dex-Verapamil, Dipyridamole, Pimozide, Haloperidol, Chlorpromazine, Trifluoperazine, Fluphenazine, Reserpin, Clopenthixol, Flupentixol, N-acetyldaunorubicin, Vindoline, N2762-14, N276-14, N276-17, B9309-068, BIBW-22, Carvedilol, Clofazimine, Ketoconazole, Lovastatin, N-Norgallopamil, Simvastatin, Troleandomycin, Vinblastin, Itraconazole, Econazole, Oligomycine, Cyclosporin and Rapamycin; and/or

(b) an inhibitor of a member of the ABCA subfamily selected from Glyburide, DIDS (4,4-diisothiocyanatostilbene-2,2-disulfonic acid), Bumetanide, Furosemide, Sulfobromophthalein, Diphenylamine-2-carboxylic acid and Flufenamic acid; and/or

(c) an inhibitor of a member of the human ABC-C (MRP)-subfamily selected from MK-571, Benzbromaron, PAK-104P, Probenecid, Sulfinpyrazone, Indomethacin, Merthiolate and Ethacrynic acid; and/or

(d) (an) antibody(ies) or functional fragments thereof which is(are) specifically recognizing one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer; and/or

(e) (an) antisense oligomere(s), iRNA and/or siRNA directed against one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer; and/or

(f) (an) aptamer(s) directed against one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer.

8. The use of any one of claims 1 to 7, wherein said arthritis is characterized by a degeneration and/or a destruction of cartilage.

9. The use of any one of claims 1 to 8, wherein said arthritis is osteoarthritis, (juvenile) chronic arthritis, rheumatoid arthritis, psoriatic arthritis, *A. mutilans*, septic arthritis, infectious arthritis and/or reactive arthritis.

10. The use of any one of claims 1 to 9, wherein said inhibitor(s) is(are) to be administered prophylactically.

11. The use of any one of claims 1 to 9, wherein said inhibitor(s) is(are) to be administered therapeutically.

12. A method for screening a compound which is suitable for the treatment of arthritis, said method comprising:

(a) contacting an isolated lipid bilayer comprising at least one ABC-transporter which is capable of transporting hyaluronan with a test compound and an indicator compound;

(b) measuring the effect of the test compound on the transport of the indicator compound across the lipid bilayer; and

(c) identifying test compounds which reduce the transport of the indicator compound.

13. A method for screening a compound which reduces the transport of hyaluronan mediated by (an) ABC-transporter(s), said method comprising:

(a) contacting an isolated lipid bilayer comprising at least one ABC-transporter which is capable of transporting hyaluronan with a test compound and an indicator compound;

(b) measuring the effect of the test compound on the transport of the indicator compound across the lipid bilayer; and

(c) identifying test compounds which reduce the transport of the indicator compound.

14. A method of screening for a compound which is suitable for the treatment of arthritis, said method comprising:

(a) contacting a cell comprising at least one ABC-transporter which is capable of transporting hyaluronan with a test compound and an indicator compound;

(b) measuring the effect of the test compound on the transport of the indicator compound across a lipid bilayer of the cell; and

(c) identifying compounds which reduce the transport of the indicator compound.

15. A method for screening a compound which reduces the transport of hyaluronan mediated by (an) ABC-transporter(s), said method comprising:

(a) contacting a cell comprising at least one ABC-transporter which is capable of transporting hyaluronan with a test compound and an indicator compound;

(b) measuring the effect of the test compound on the transport of the indicator compound across a lipid bilayer of the cell; and

(c) identifying compounds which reduce the transport of the indicator compound.

16. The method of any one claim 12 to 15 for screening a compound which specifically reduces the transport of hyaluronan mediated by said ABC-transporter.

17. The method of any one of claims 14 to 16, wherein the cell is a bacterial, an insect, a fungal or an animal cell.

18. The method of claim 17, wherein said animal cell is a mammalian cell or a mammalian cell line.

19. The method of claim 18, wherein said mammalian cell or mammalian cell line is derived from human, horse, swine, goat, cattle, mouse or rat.

20. The method of claim 18 or 19, wherein the cell or cell line is a chondrocyte, a fibroblast, a synovial cell, an endothelial cell, a macrophage, a tumour cell or a smooth muscle cell, melanoma cell or mesotheliama cell.

21. The method of claim 20, wherein said cell is comprised in a tissue.

22. The method of claim 21, wherein said tissue is cartilage tissue.

23. The method of any one of claims 18 to 22, wherein said cell or said tissue is derived from a mammalian subject preferably a human subject which suffers from arthritis.

24. The method of any one of claims 18 to 22, wherein the cell comprises at least one heterologous ABC-transporter.

25. The method of any one of claims 18 to 24, wherein said cell and/or said tissue is comprised in a non-human animal.

26. The method of any one of claims 14 to 24 which is *ex vivo*.

27. A method of screening for a compound which is suitable for the treatment of arthritis in a subject, said method comprising:

(a) contacting a cell derived from said subject which comprises at least one ABC-transporter with a test compound to be tested;

(b) measuring the effect of the test compound on the transport of an indicator compound across a lipid bilayer of said cell; and

(c) identifying compounds which reduce the transport of hyaluronan across the lipid bilayer of said cell.

28. The method of claim 27, wherein said cell is comprised in a tissue.

29. The method of any one of claims 27 to 28, wherein said cell is a chondrocyte.

30. The method of any one of claims 27 to 29, wherein said subject is a mammalian subject.

31. The method of claim 30, wherein said mammalian subject is a human, a horse, a camel, a dog, a cat, a pig, a cow, a goat or a fowl.

32. The method of any one of claims 27 to 31, wherein said cell is contacted with a compound selected from the group consisting of:

(a) an inhibitor of a member of the ABCB (MDR)-subfamily selected from Verapamil, Valspodar (PSC833), Elacridar (GF-120918), Bericodar (VX-710), Tariquidar (XR-9576), XR-9051, S-9788, LY-335979, MS 209, R101933; OC-144-093; Quinidine, Chloripramine, Nicardipine, Nifedipine, Amlodipine, Felodipine, Manidipine, Flunarizine, Nimodipine, Pimozide, Lomerizine, Bepridil, Amiloride, Almitrine, Amiodarone, Imipramine, Clomiphene, Tamoxifen, Toremifene, Ketocanazole, Terfenadine, Chloroquine, Mepacrin, Diltiazem, Niguldipine, Prenylamine, Gallopamil, Tiapamil, Dex-Verapamil, Dipyridamole, Pimozide, Haloperidol, Chlorpromazine, Trifluoperazine, Fluphenazine, Reserpin, Clopenthixol, Flupentixol, N-acetyldaunorubicin, Vindoline, N2762-14, N276-14, N276-17, B9309-068, BIBW-22, Carvedilol, Clofazimine, Ketoconazole, Lovastatin, N-Norgallopamil, Simvastatin, Troleandomycin, Vinblastin, Itraconazole, Econazole, Oligomycine, Cyclosporin and Rapamycin; and/or

(b) an inhibitor of a member of the ABCA subfamily selected from Glyburide, DIDS (4,4-diisothiocyanatostilbene-2,2-disulfonic acid), Bumetanide, Furosemide, Sulfobromophthalein, Diphenylamine-2-carboxylic acid and Flufenamic acid; and/or

(c) an inhibitor of a member of the human ABC-C (MRP)-subfamily selected from MK-571, Benzbromaron, PAK-104P, Probenecid, Sulfinpyrazone, Indomethacin, Merthiolate and Ethacrynic acid; and/or

(d) (an) antibody(ies) or functional fragments thereof which is(are) specifically recognizing one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer; and/or

(e) (an) antisense oligomere(s), iRNA and/or siRNA directed against one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer; and/or

(f) (an) aptamer(s) directed against one or more ABC-transporter(s) capable of transporting hyaluronan across a lipid bilayer.

33. The method of any one of claims 12 to 31, wherein the test compound is a small molecule or a peptide derived from an at least partially randomised peptide library.

34. The method of any one of claims 12 to 33 further comprising a step of refining the compound identified, said method comprising the steps of:

(a) identification of the binding sites of the compound and the ABC-transporter(s);

(b) molecular modelling of the binding site of the compound; and

(c) modification of the compound to improve its binding specificity for the ABC-transporter(s).

35. The method of claim 34, wherein said compound is further refined by peptidomimetics.

36. The method of any one of claims 12 to 35 further comprising the step of modifying the compound identified or refined as a lead compound to achieve:

(a) modified site of action, spectrum of activity, organ specificity, and/or

(b) improved potency, and/or

(b) decreased toxicity (improved therapeutic index), and/or

(d) decreased side effects, and/or

(e) modified onset of therapeutic action, duration of effect, and/or

(f) modified pharmakinetic parameters (resorption, distribution, metabolism and excretion), and/or

(g) modified physico-chemical parameters (solubility, hygroscopicity, color, taste, odor, stability, state), and/or

(h) improved general specificity, organ/tissue specificity, and/or

(i) optimized application form and route by:

(i) esterification of carboxyl groups, or

(ii) esterification of hydroxyl groups with carbon acids, or

(iii) esterification of hydroxyl groups to, e.g. phosphates, pyrophosphates or sulfates or hemi succinates, or

(iv) formation of pharmaceutically acceptable salts, or

(v) formation of pharmaceutically acceptable complexes, or
(vi) synthesis of pharmacologically active polymers, or
(vii) introduction of hydrophilic moieties, or
(viii) introduction/exchange of substituents on aromates or side chains, change of substituent pattern, or
(ix) modification by introduction of isosteric or bioisosteric moieties, or
(x) synthesis of homologous compounds, or
(xi) introduction of branched side chains, or
(xii) conversion of alkyl substituents to cyclic analogues, or
(xiii) derivatisation of hydroxyl group to ketales, acetales, or
(xiv) N-acetylation to amides, phenylcarbamates, or
(xv) synthesis of Mannich bases, imines, or
(xvi) transformation of ketones or aldehydes to Schiff's bases, oximes, acetales, ketales, enolesters, oxazolidines, thiozolidines

or combinations thereof.

37. The method of any one of claims 12 to 36, further comprising the step of formulating the compound identified, refined or modified with a pharmaceutically active carrier and/or diluent.

38. A method for manufacturing a pharmaceutical composition comprising the steps of any one of claims 12 to 37 and the step of formulating the compound screened in a pharmaceutically acceptable form.

39. A method of preventing, ameliorating and/or treating the symptoms of arthritis in a subject comprising administering at least one inhibitor of at least one ABC-transporter capable of transporting hyaluronan across a lipid bilayer to the subject such that the arthritis is prevented, ameliorated and/or treated.

40. The method of claim 39, wherein said arthritis is characterized by a degeneration and/or a destruction of cartilage.

41. The method of claim 39 or 40, wherein said arthritis is osteoarthritis, (juvenile) chronic arthritis, rheumatoid arthritis, psoriatic arthritis, *A. mutilans*, septic arthritis, infectious arthritis and/or reactive arthritis.

43. The method of any one of claims 40 to 42, wherein said mammalian subject is a human, a horse, a camel, a dog, a cat, a pig, a cow, a goat or a fowl.

Abstract

The present invention relates to the use of at least one inhibitor of at least one ABC-transporter capable of transporting hyaluronan across a lipid bilayer, for the preparation of a pharmaceutical composition for the treatment of arthritis. Furthermore, the present invention relates to a method for screening a compound which is suitable for the treatment of arthritis. The present invention also relates to a method for screening a compound which reduces the transport of hyaluronan mediated by (an) ABC-transporter(s). Furthermore, the present invention relates to a method for identifying a subject at risk for arthritis as well as to a method of screening for a compound which is suitable for the treatment of arthritis in a subject. In addition, the present invention relates to a method of preventing, ameliorating and/or treating the symptoms of arthritis in a subject.

Figure 1

48 Human ATP-Binding Cassette Transporters

Name	ABC1	MDR	MRP	ALD	OABP	GCN20	White
Subfamily	ABCA	ABCB	ABCC	ABCD	ABCE	ABCF	ABCG
Members	12	11	12	4	1	3	5 (+1?)
Some Inhibitors	DIDS Glyburide	Verapamil Valspodar Nicardipin Nimodipin	MK-571 Benzbromaron				

Symbols Access						
ABCA1: NM005502	ABCB1 NM000927	ABCC1 NM 004996	ABCD1 NM 000033	ABCE1 NM 002940	ABCF1 NM 001090	ABCG1 NM 004915
ABCA2: NM001606	ABCB2 NM000593	ABCC2 NM 000392	ABCD2 NM 005164		ABCF2 NM 005692	ABCG2 NM 004827
ABCA3 NM001089	ABCB3 NM000544	ABCC3 NM 003786	ABCD3 NM 002858		ABCF3 AK002060	ABCG3
ABCA4 NM000350	ABCB4 NM000443	ABCC4 NM 005845	ABCD4 NM 005050			ABCG4 NM 022169
ABCA5 NM018672	ABCB5 U66692	ABCC5 NM 005688				ABCG5 NM 022436
ABCA6 NM080284	ABCB6 NM 005689	ABCC6 NM 001171				ABCG8 NM 022437
ABCA7 NM019112	ABCB7 NM004299	ABCC7 NM 000492				
ABCA8 NM007168	ABCB8 NM 007188	ABCC8 NM 000352				
ABCA9 NM080283	ABCB9 NM 019624	ABCC9 NM 005691				
ABCA10 NM080282	ABCB10 NM 012089	ABCC10 XM052745				
ABCA12 NM015657	ABCB11 NM 003742	ABCC11 NM 033151				
ABCA13		ABCC12 NM 033226				

Figure 2

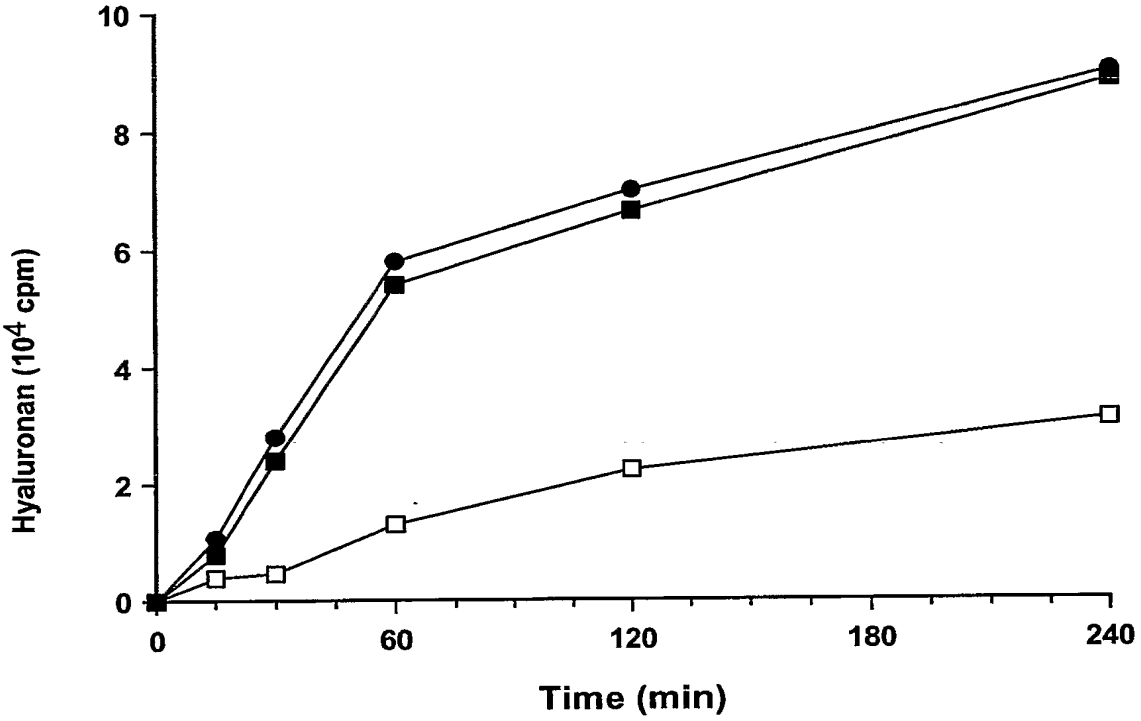
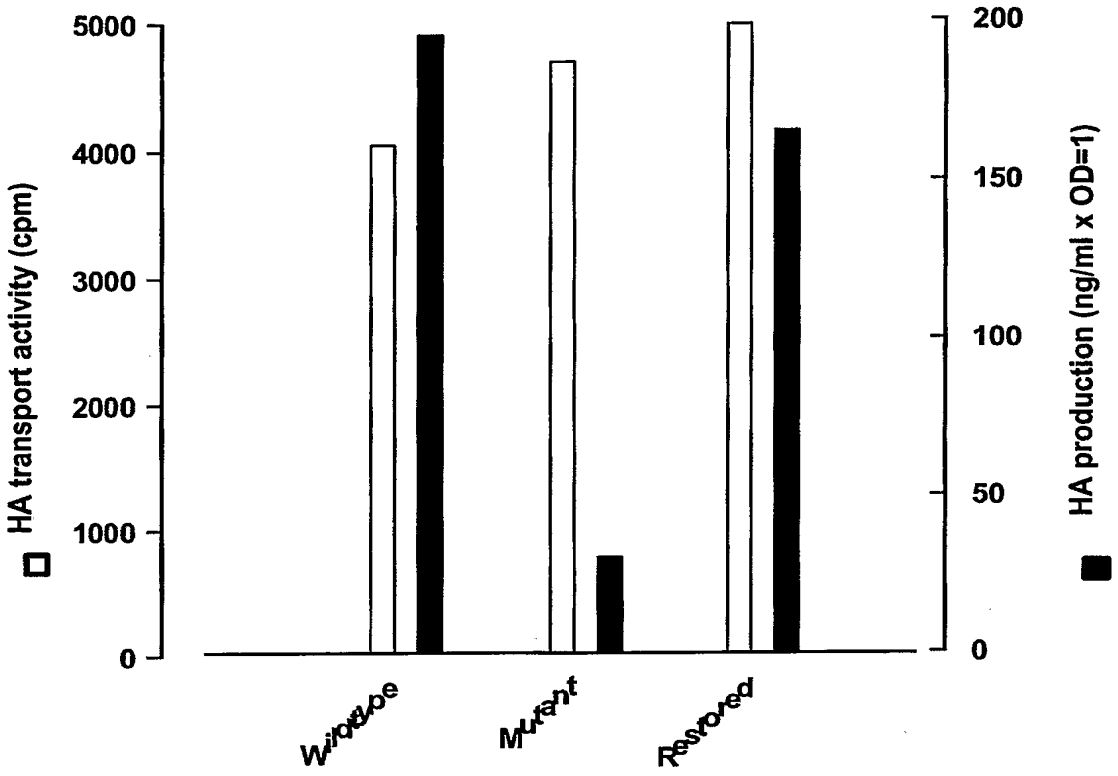
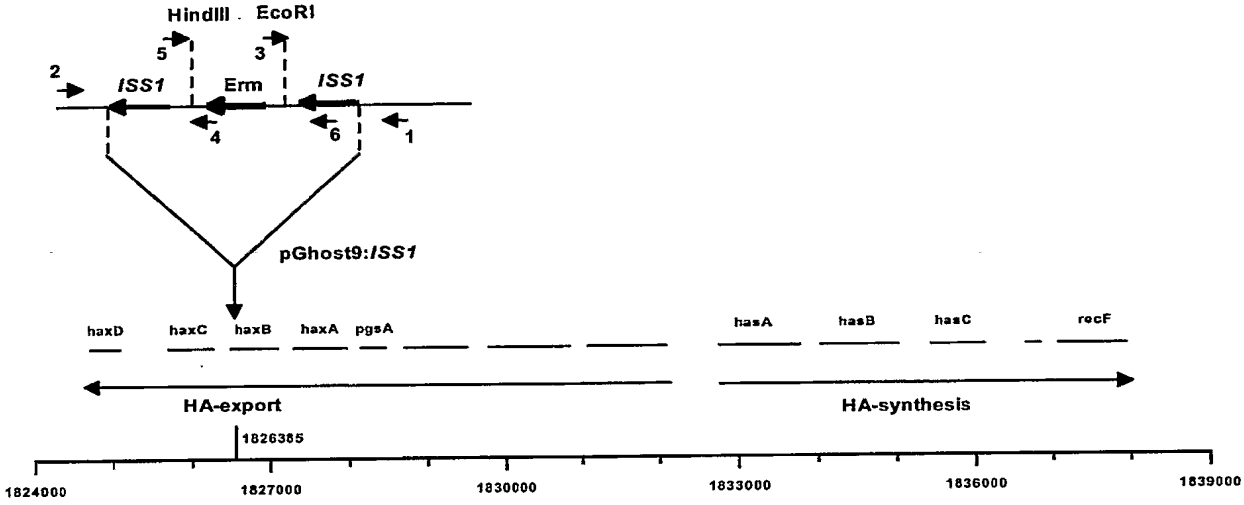


Figure 3


5000
4000
3000
2000
1000
0
HA transport activity (cpm)
200
150
100
50
0
HA production (ng/ml x OD=1)
Wildtype
Mutant
Restored

5

Figure 4


HindIII
EcoR1
5
3
2
ISS1
Erm
ISS1
4
6
1
pGhost9:ISS1
haxD
haxC
haxB
haxA
pgsA
hasA
hasB
hasC
recF
HA-export
HA-synthesis
1826385
1824000
1827000
1830000
1833000
1836000
1839000
Location

5

Figure 5

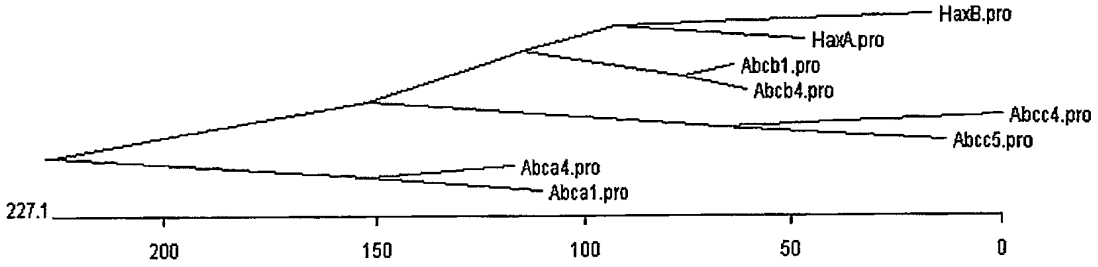
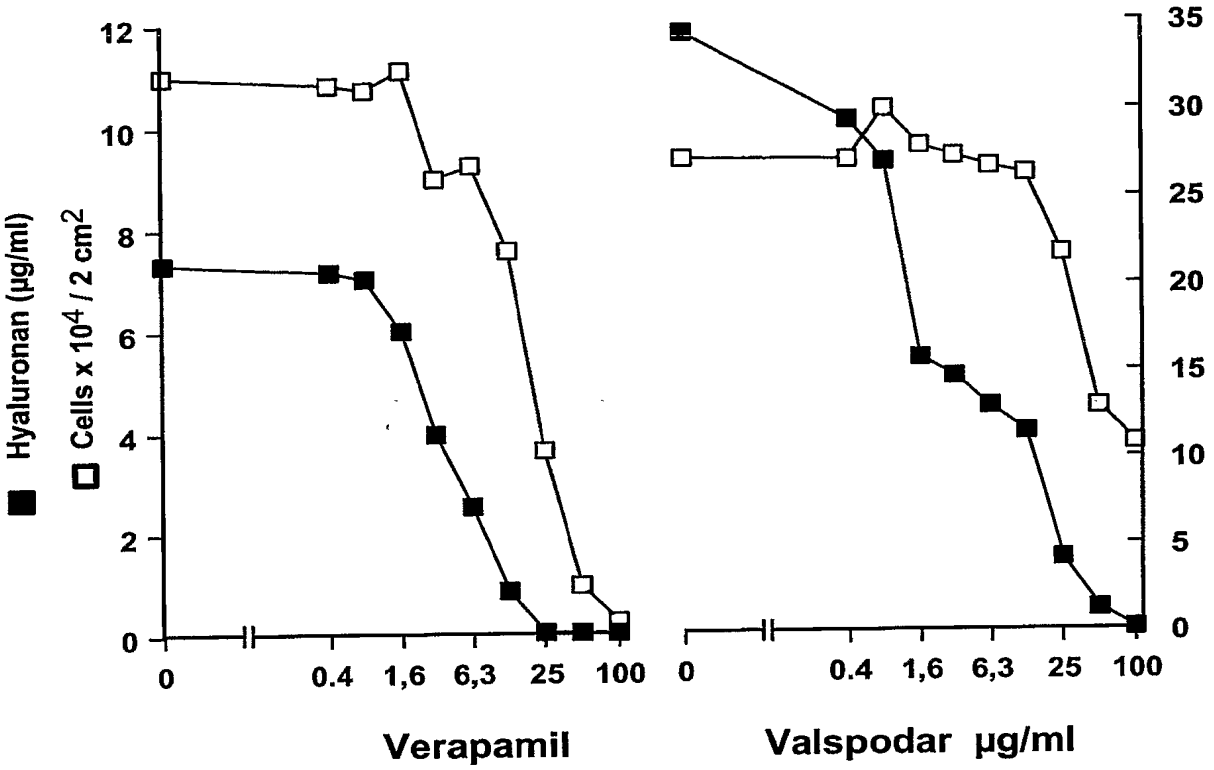

HaxB.pro
HaxA.pro
Abcb1.pro
Abcb4.pro
Abcc4.pro
Abcc5.pro
Abca4.pro
Abca1.pro
227.1
200
150
100
50
0

Figure 6


Hyaluronan (µg/ml)
Cells x 10^4 / 2 cm^2
12
10
8
6
4
2
0
0
0.4
1,6
6,3
25
100
Verapamil
35
30
25
20
15
10
5
0
0
0.4
1,6
6,3
25
100
Valspodar µg/ml

5

Figure 7

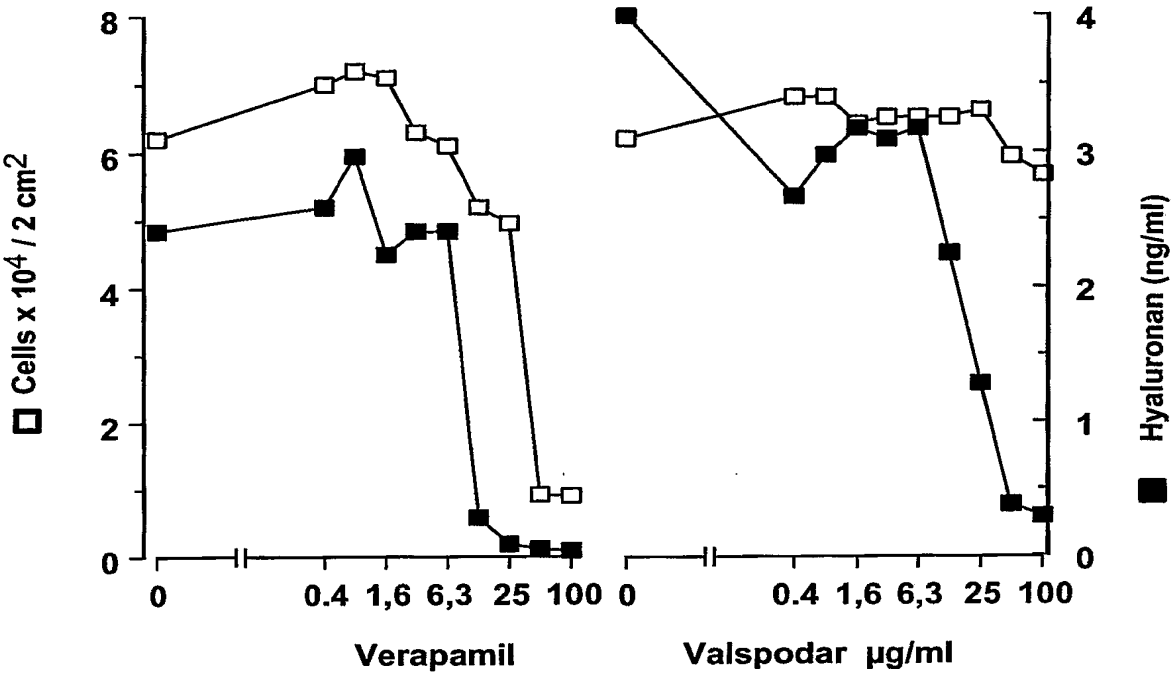
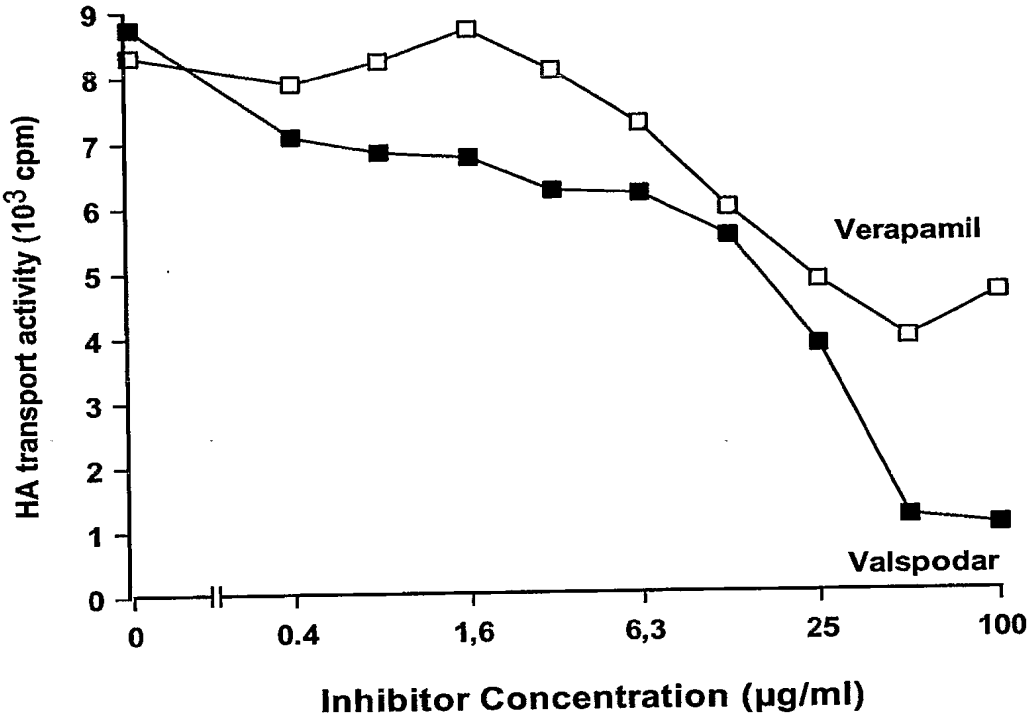

Cells x 10^4 / 2 cm^2
Hyaluronan (ng/ml)
0
2
4
6
8
0
1
2
3
4
0
0.4
1,6
6,3
25
100
Verapamil
Valspodar µg/ml

Figure 8


HA transport activity (10^3 cpm)
9
8
7
6
5
4
3
2
1
0
Verapamil
Valspodar
0
0.4
1,6
6,3
25
100
Inhibitor Concentration (µg/ml)

5

Figure 9

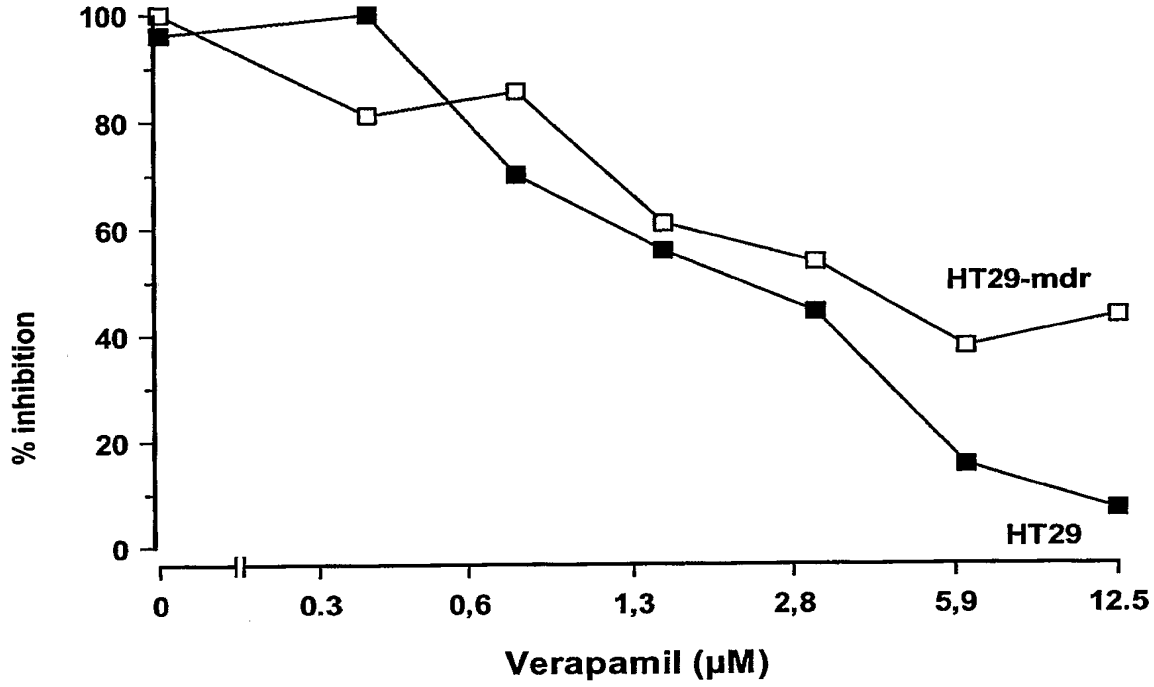
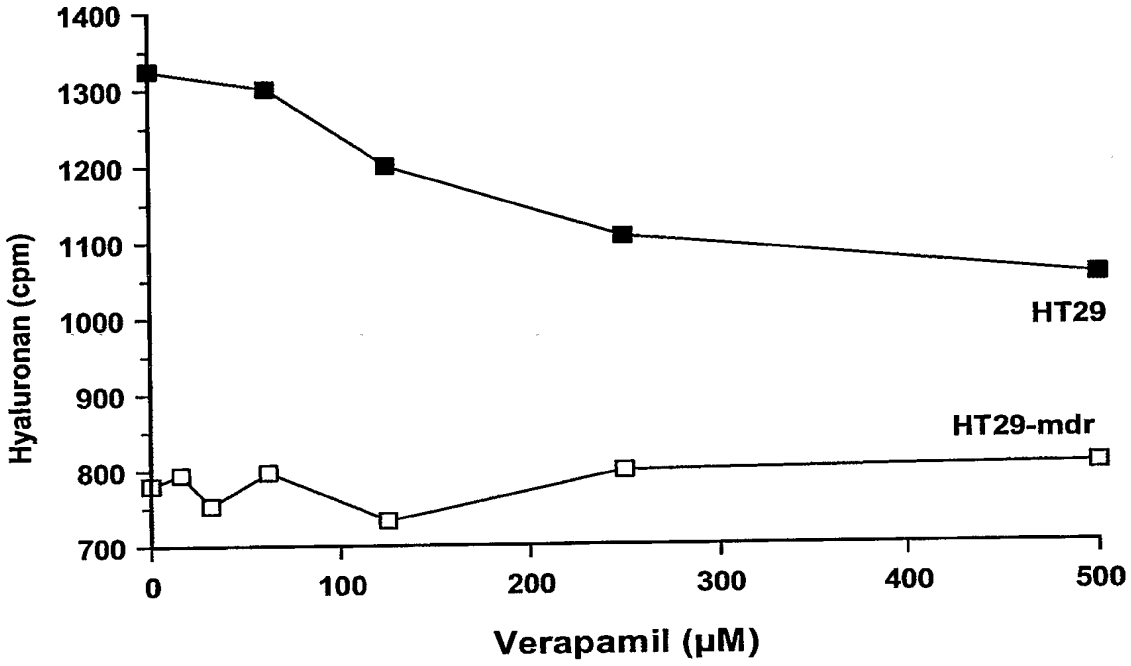

100
80
60
40
20
0
% inhibition
HT29-mdr
HT29
0
0.3
0,6
1,3
2,8
5,9
12.5
Verapamil (µM)

Figure 10


1400
1300
1200
1100
1000
900
800
700
Hyaluronan (cpm)
HT29
HT29-mdr
0
100
200
300
400
500
Verapamil (µM)

5

Figure 11

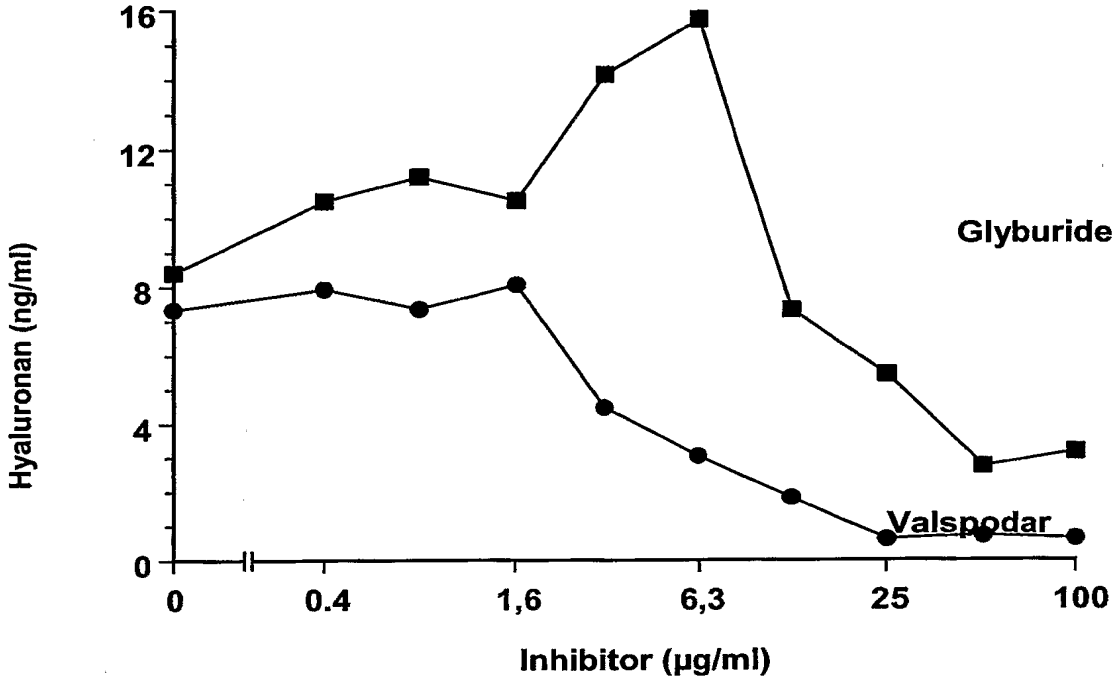
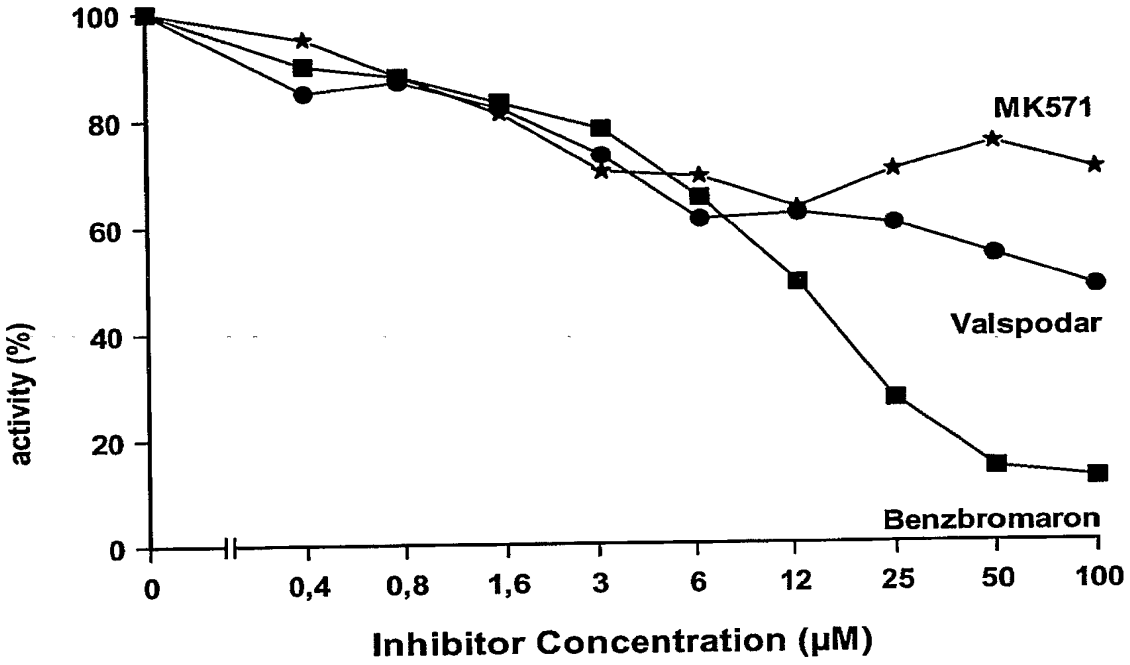
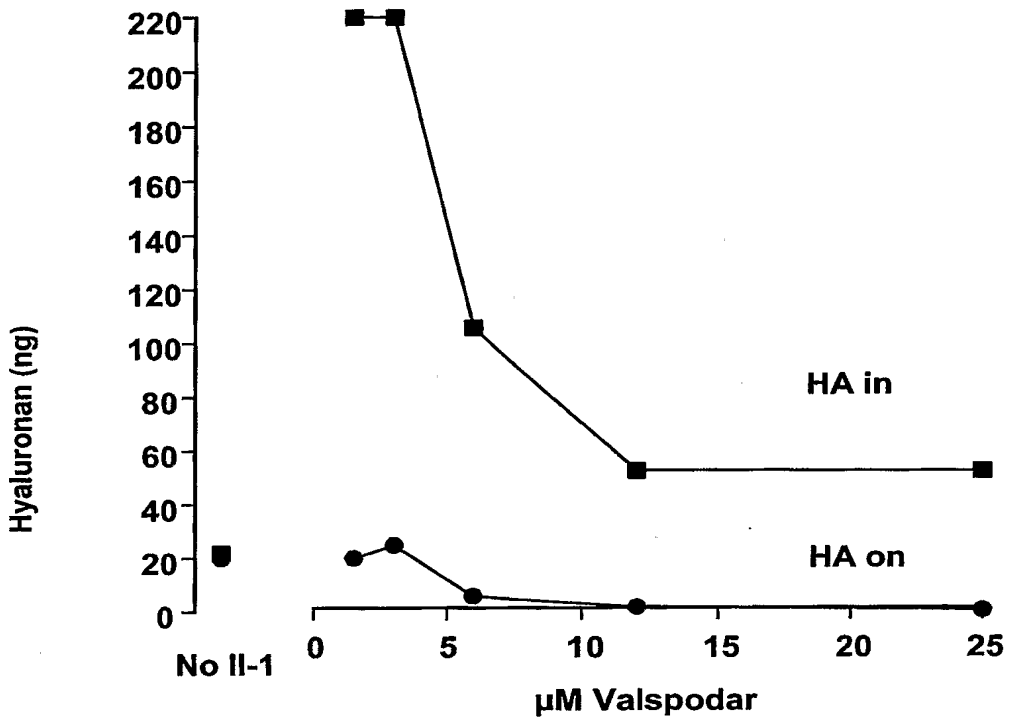

16
12
8
4
0
Hyaluronan (ng/ml)
Glyburide
Valspodar
0
0.4
1,6
6,3
25
100
Inhibitor (µg/ml)

Figure 12


100
80
60
40
20
0
activity (%)
MK571
Valspodar
Benzbromaron
0
0,4
0,8
1,6
3
6
12
25
50
100
Inhibitor Concentration (µM)

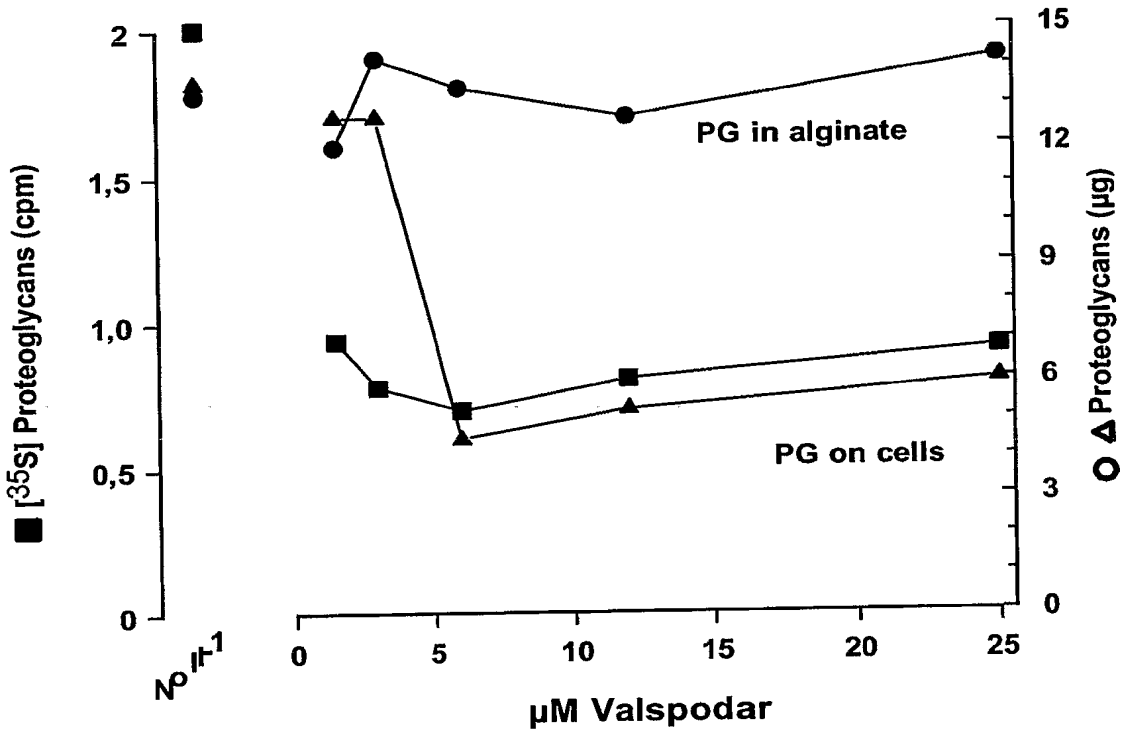
5

Figure 13


Hyaluronan (ng)
220
200
180
160
140
120
100
80
60
40
20
0
No Il-1
HA in
HA on
0
5
10
15
20
25
µM Valspodar

5

Figure 14


2
1,5
1,0
0,5
0
■ [35S] Proteoglycans (cpm)
No h-1
0
5
10
15
20
25
µM Valspodar
15
12
9
6
3
0
O Δ Proteoglycans (µg)
PG in alginate
PG on cells

5

Figure 15

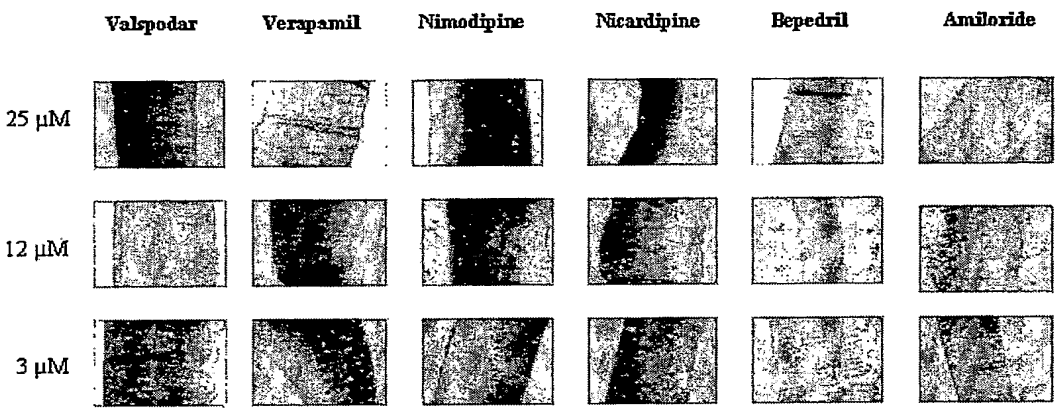

Valspodar
Verapamil
Nimodipine
Nicardipine
Bepedril
Amiloride
25 µM
12 µM
3 µM

Figure 16

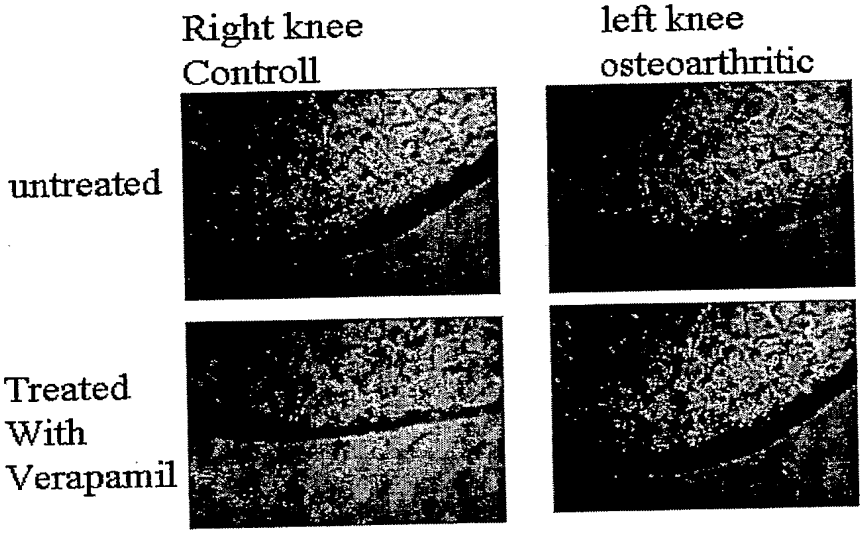

Right knee
Controll
left knee
osteoarthritic
untreated
Treated
With
Verapamil

Figure 17